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Synergism of Indole and Indole-3-acetic Acid in the Root Production of Phaseolus Cuttings

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1. Introduction

An insight into the activity of the synergists was generally thought important for the problem of the relation between the molecular structure and activity of the auxins. The synergism of indole and IAA in the rooting of petioles of *Ageratum* (van Raalte, 4) led to the suggestion that the enhancing effect of indole on the IAA activity was caused by its decreasing effect on the IAA oxydase. He showed that enzyme extracts of etiolated pea seedlings oxidized IAA and that this reaction was decreased by the addition of indole. Moreover the *Ageratum* petioles reacted also to NAA by greater root production, but this reaction was not increased by indole. These results suggest that the synergism of indole and IAA is caused by a decreased IAA oxydation. Oxydation of NAA does not seem to take place, or at least is not influenced by indole.

The similarity in molecular structure of IAA and indole seems to indicate that the substances are attached to the enzymes by that part of the molecule that is common to both: the indole nucleus. The indole molecules may block certain positions on the oxidizing enzyme, preventing the IAA molecules to occupy these positions, with the consequence that oxidation decreases and the activity of IAA becomes stronger. This theory is almost the same as the supposed synergism of auxins and hemi-auxins (substances which have no effect when applied alone). The effect of these substances is explained by

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the well known two-reactions theory of Went (9). In the first reaction auxin and hemi-auxin are both active and the synergist can replace the auxin; in the second reaction only the auxin is active, so that the more places on the common adsorbing surface are occupied by the synergist, the more auxin molecules are available for the second reaction. A competition of auxin and synergist molecules for the same places of adsorbing surfaces (either enzyme or some other surface layer in the cell) is the theory most usually accepted about the synergism (Skoog, Schneider and Malan, 7; Veldstra, 8). With a slight modification (the adsorbing surface belongs to an oxidizing enzyme) the results of van Raalte give a more precise indication of the synergism of indole and IAA. The presence of an IAA oxidizing enzyme was however proved in pea seedlings, not in *Ageratum* petioles whereas the synergistic effect of indole on IAA activity has only been found in the root production of *Ageratum* petioles. Therefore experiments are described here about the synergistic effect of IAA and indole could be found in other materials. In these experiments cuttings of *Phaseolus vulgaris* showed the same effect. As Hemberg (1) found that the roots of *Phaseolus* cuttings needed Boron for their growth, this element was added. The effect it exerted made it necessary to make a more thorough investigation of the activity of this element.

2. Material and Methods

Cuttings of *Phaseolus vulgaris* (var. 'Vroege Wagenaar') were used in all experiments. Seeds were sown in sawdust and fairly homogenous seedlings were obtained. The hypocotyls were cut either 5 cm, below the cotyledons (these were then removed) or directly above the cotyledons. (The last method proved to be the better, the wounds of the cut cotyledons forming entrances for infections). The cuttings were placed 2 cm deep in the solutions and remain there for 24 hours. Indole and indole-3-acetate (K) were dissolved in a buffer solution containing 0.69 g KH_2PO_4 + 0.05 g CaCl_2 per liter distilled water. After 24 hours in the solution the cuttings were transferred to tap water with addition of H_3BO_3 10 p.p.m. and CuSO_4 0.1 p.p.m. The first compound was added because Hemberg (1) found that boron is necessary for root formation and the second compound as a disinfectant.

Indole was used in the concentrations 10^{-3} and 10^{-5} M; K-indole-3-acetate in the concentrations 10^{-7} , 10^{-6} , 10^{-5} and 10^{-4} M. After 10 to 15 days the number of roots was recorded.

3. The Effect of Indole

In Table 1 and Figure 1 the results of a typical experiment on the indole effect are summarized.

The following conclusions may be drawn:

Table 1. *Rooting of cuttings under the influence of IAA and Indole in various concentrations. Root production as a percentage of the control.*

Indole M	K-IAA M				
	0	10^{-7}	10^{-6}	10^{-5}	10^{-4}
0	100	122	130	185	327
10^{-5}	137	135	154	198	316
10^{-3}	166	161	255	312	468

a. In the concentrations 10^{-7} to 10^{-4} M IAA the root production increases with increasing concentration.

b. Indole enhances the effect of all concentrations of IAA.

c. Indole 10^{-3} M has a stronger effect than 10^{-5} M.

d. Indole alone has a stimulating effect on root production. This last statement is unexpected and not in accordance with the results of van Raalte with *Ageratum* petioles. The difference however, is easily explained when we assume that the *Ageratum* petioles do not possess so much endogenous auxin as the *Phaseolus* stems.

e. The effect of indole 10^{-5} M alone is the same as IAA 10^{-6} M alone (137 and 130, resp., see table 1) and the effect of indole 10^{-3} M as 10^{-5} M IAA.

This shows that the activity of indole is rather high. Indole (in certain concentrations) is only 10 times less active than IAA.

The enhancing effect of indole (10^{-3} M) can also be seen in figures 3 and 6, on comparing the curves indole 10^{-3} M + B 10^{-6} M with B 10^{-6} M. It can be deduced from these curves that:

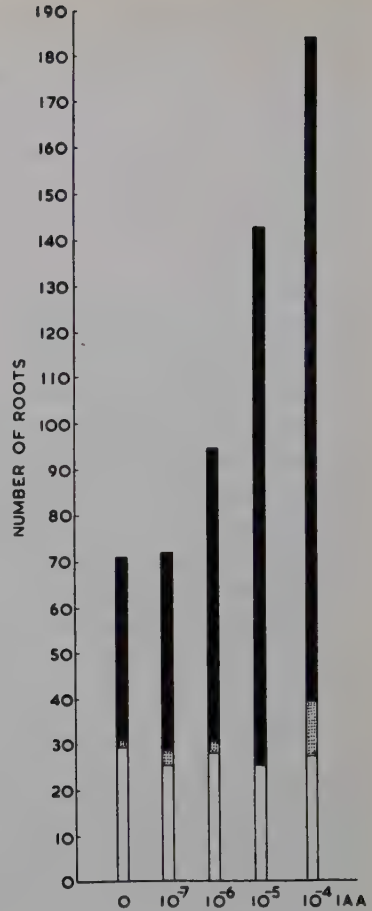
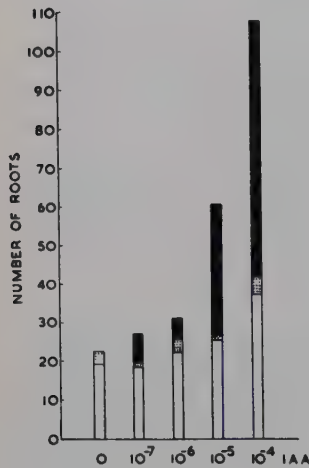
a. Root production increases with the logarithm of the IAA concentration (straight line, "control" in figure 3).

b. When indole is added the semilogarithmic and the double logarithmic curves are not straight lines, but become convex to the x-axis, which means that the increase of root production with increasing IAA concentration is more than logarithmic. For the explanation of this "indole-effect" in connection with a decreased oxydase activity, see the discussion.

In a number of similar experiments IAA had no influence on root production. Indole, however, applied together with the IAA had a stimulating influence. The stimulating effect became stronger as the (*per se* inactive) IAA concentration increased. This is shown in Figure 2. A possible explanation of these findings is as follows: In the cases where the IAA has no influence, the plant might contain a high amount of IAA oxydase, which enzyme inactivates the IAA. When adding indole, part of the oxydase is blocked by the indole and the IAA effect shows up.

Figure 2. Root production of *Phaseolus* cuttings with IAA and indole. Indications the same as in figure 1. IAA in this experiment inactive. Indole 10^{-3} M synergistic with the inactive IAA.

Figure 1. Root production of *Phaseolus* cuttings with IAA and indole. White column: IAA; stippled column: the increase by indole 10^{-5} M; black column: the increase by indole 10^{-3} M.



The amount of IAA oxydase that is inactivated or blocked by indole 10^{-5} M should be roughly the same as the quantity that oxidizes IAA 10^{-6} M.

4. The Effect of Boron

Among the inorganic compounds influencing root production boron seems to be the most important (Hemberg, 1).

Therefore, boron was added to the rooting solutions. The effect can be seen in Figure 3. In the course of the experiments it was noticed that the boron concentration was important and for that reason some concentrations were compared. Figures 4 and 5 give the results of these experiments. It is

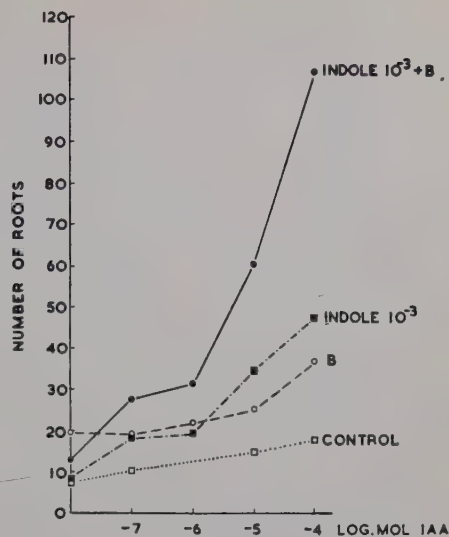


Figure 3. The effect of indole and of B and both substances together on the rooting effect of IAA.

Control	= IAA alone.
B	= IAA + B 10^{-6} M.
Indole 10^{-3}	= IAA + Indole 10^{-3} M.
Indole 10^{-3} + B	= IAA + Indole 10^{-3} M + B 10^{-6} M.

evident that the concentration 10^{-6} M is better than 10^{-3} M , which is supra-optimal.

The results of these experiments show that:

a. B increases root production with a constant factor. This holds true as well for IAA in all concentrations as for IAA+indole.

b. Of the investigated concentrations (6.1×10^{-5} and 6.1×10^{-8} per cent = 10^{-3} and 10^{-6} M) 10^{-6} M is optimal. 10^{-3} M has a damaging effect (leaves

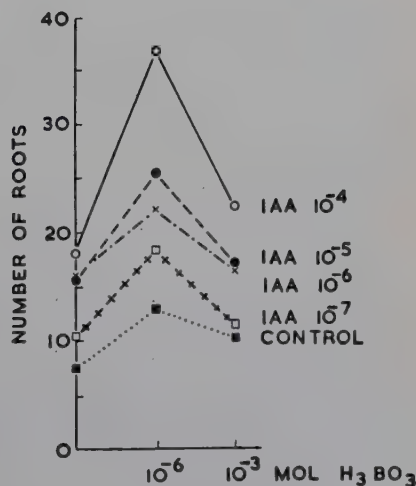


Figure 4. Influence of different B concentration on the rooting effect of IAA.

Control=no IAA.
IAA conc. in *M*.

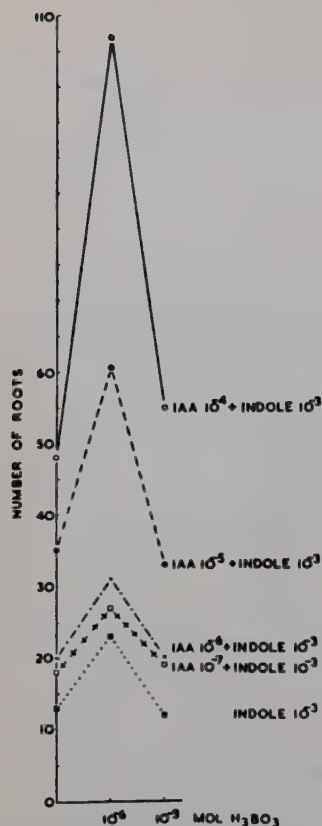
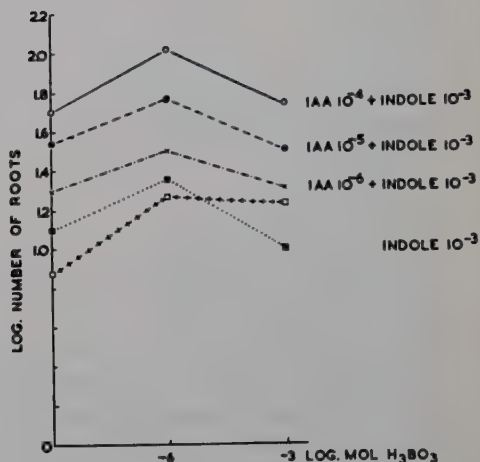


Figure 5. Influence of different B concentrations on the rooting effect of IAA + Indole. All concentrations in M.



are abscised). The effect of boron 10^{-6} M is rather large (increase to 100 per cent).

In figure 6 these findings are shown in double logarithmic curves. The regularity is striking. The curves are as parallel as is possible for any physiological experiment. In all cases boron increases the root production with a logarithmically similar number, it raises every curve to a higher level.

From this it is evident that boron does not exert its influence in the same processes as indole, because this compound changes the straight line into a convex one. According to both Rehm (6) and Hemberg (1) it is the growth of the roots which is influenced by boron, not the initiation of the root primordia. This theory does not contradict the above mentioned results, which are best explained by assuming that indole decreases the activity of the IAA oxydase, while boron stimulates the development of the roots from the primordia.

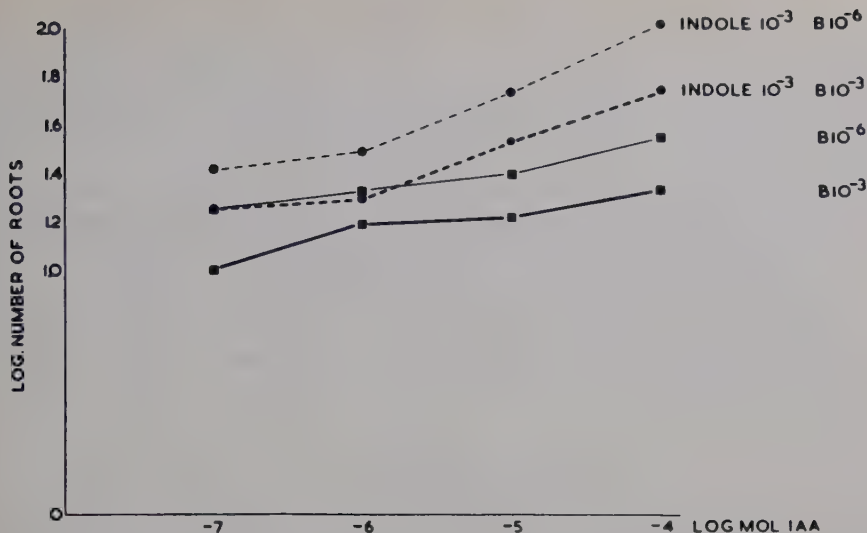


Figure 6. Synergism of IAA and indole and the influence of B. The effect of B is a logarithmically straight line. The effect of indole is represented by a curve convex to the x-axis.

5. Discussion

In the foregoing experiments some points are of special interest.

Firstly: In most of the experiments root production increased with increasing IAA concentrations. The relation is represented by a straight line when root number is plotted against the logarithm of concentration (see Figure 3, control). In some experiments however IAA does not have any effect on root production. The explanation for this difference seems to be that the plant can contain such large quantities of IAA oxydase that all applied IAA is inactivated.

Secondly: The "indole effect". In *Phaseolus* indole enhances root production as well together with IAA as alone. A most striking result is, that the logarithmic straight line of the relation root number — IAA concentration (see Figure 3) is changed by the indole into a curve that is convex to the x-axis. This "indole-effect" cannot be explained by the blocking of a certain quantity of IAA oxydase by indole. For in that case the logarithmic curve would remain a straight line parallel to the normal IAA concentration curve, only running on a higher level. The curve however becomes convex. A possible explanation is, that IAA oxydase is an adaptive enzyme the concentration of which increases with IAA concentration. A necessary

assumption is then that indole blocks a constant percentage of the IAA oxydase and that this part is less than $1/10$.

Thirdly: In some experiments IAA did not influence root production. Nevertheless indole added to the IAA enhanced the root production and its effect became stronger as the IAA concentration increased.

In these cases the plant must contain such large quantities of IAA oxydase that whatever concentration of IAA is added, it has no effect. When part of the IAA oxydase is blocked by indole, the effect of differences in concentration of IAA become apparent.

As indole 10^{-5} M has the same effect as IAA 10^{-6} M alone, the quantity of IAA oxydase that is blocked by indole 10^{-5} M must be that quantity that oxidizes IAA 10^{-6} M.

6. Summary

1. The synergism of indole and IAA was investigated by recording the production of adventitious roots of hypocotyls of *Phaseolus vulgaris*.

2. IAA in concentrations 10^{-7} to 10^{-4} M increases root production (the relation: number of roots — log IAA conc. is a straight line).

3. Indole (10^{-3} M and 10^{-6} M) enhances the effect of IAA in all concentrations. The curve: number of roots — log IAA concentration becomes convex to the x-axis. This "indole effect" can be explained by the assumption that indole decreases the IAA oxydase activity and that this oxydase is an adaptive enzyme.

4. Boron increases root production, as well in combination with IAA as with IAA+indole. In all cases boron brings the root production to a higher level, never altering the shape of the curves.

5. It was suggested that:

a. Indole decreases the IAA oxydase activity.

b. Boron influences the growth in length of the roots.

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Studies on the Physiological Effect of Gibberellin II.

On the Interaction of Gibberellin with Auxins and Growth Inhibitors

By

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Brian *et al.* (1) considered gibberellic acid to be a kind of auxin according to the definition of Tukey *et al.* (3). The present author, on the other hand, reported that gibberellin was a substance quite different from indole-3-acetic acid in its mechanism of action (2). Hence if gibberellin should be listed as a member of auxin by the definition of Tukey *et al.* (3), it should be put in a class different from the known auxin substances.

In order to confirm that the site of action is different between gibberellin and auxin, interaction between the two substances was studied. This paper reports the results. The abbreviations GB, IAA, NAA, CM and MH stand for gibberellin, indole-3-acetic acid, naphthalene-acetic acid, coumarin and maleic hydrazide, respectively.

Materials and Methods

Pea stem sections were used in order to determine the interaction between GB and auxin in auxin-induced growth. Stem sections were cut from the third internode, 15—20 mm. in length, which were grown in the dark room at 25°C. for 7 days. Their initial length of section was 5.26 ± 0.14 mm. Sections were floated in the test solution for 24 hours and their final length was measured under a low power binocular microscope with an ocular micrometer.

Seedling of cucumber (*Cucumis sativus* L.) was used as material in experiments on shoot and root growth. Cucumber seed was sterilized with 0.1 per cent HgCl_2 , rinsed, and germinated in a petri dish containing filter paper moistened with dis-

tilled water. The root grew to about 2 mm. in length within 24 hours. No shoot development was observed during this period. Ten seedlings each were put in a petri dish which was lined with filter paper moistened with 6 ml. of test solution. Each test was repeated twice. The length of shoots and roots were measured after 48 hours incubation.

In the experiment on lateral bud growth, red kidney bean (*Phaseolus vulgaris* L.) was used. Seedlings were grown in the greenhouse until the first trifoliates had fully developed. The stem was decapitated 1 cm. above the lateral bud, and the cut surface treated with lanolin paste containing GB and auxin. The length of lateral bud was measured 8 days after treatment.

In the experiment on root formation, Alaska peas (*Pisum sativus* L.) were planted in moist sand and grown for 7 days in a dark room at 25°C. Under these conditions the epicotyles grew to a length of 10—12 cm. They carried two leaf scales. Epicotyl was cut from the seedling just above the first scale and then decapitated 5 mm. below the terminal bud. The lower end was treated for 15 hours with solution of GB and IAA mixed in various proportions, and then transferred to 1 per cent sucrose solution and incubated for 7 days. Finally the material was immersed in distilled water for 7 additional days, and the number of roots was counted.

GB used in this report is gibberellin A. (Supplied by Dr. Y. Sumiki, Tokyo University).

Results

Interaction of GB and auxin. Pea stem sections were treated with various concentrations of IAA and NAA with and without addition of 10 mg./l. of gibberellin. Results as represented in Figure 1 (A and B) show that GB always acted simply additively with IAA and NAA, in any of the concentrations from non-effective range to more than sufficient concentration. No synergism, nor interference, was observed.

As the cucumber seedling in a certain stage of development is more sensitive to auxin than pea stem section, it is inhibited by rather weak solution of auxin. When GB was added to auxin solution, the growth inhibition due to IAA (Table 1) and NAA (Table 2) was reduced in shoot, but not in root. GB is reported not to affect the root growth (1). Hence the results obtained can be explained as GB acting not significantly interfering with auxin. If GB were auxin, the growth inhibition by supraoptimal concentration of auxin should have been intensified both in the shoot and in the root.

Interaction between GB and growth inhibitors. GB was added to solutions of inhibitors, CM and MH. Root and shoot growth of cucumber seedling was measured. As shown in Tables 3 and 4, the inhibition of shoot growth by CM and MH was reduced by GB, but this is not the case with roots.

Effect of GB on auxin-induced bud inhibition. GB and NAA were combined in lanolin paste and applied on the cut surface of kidney bean stem. The results are shown in Table 5. The inhibiting effect of 0.1 per cent NAA was

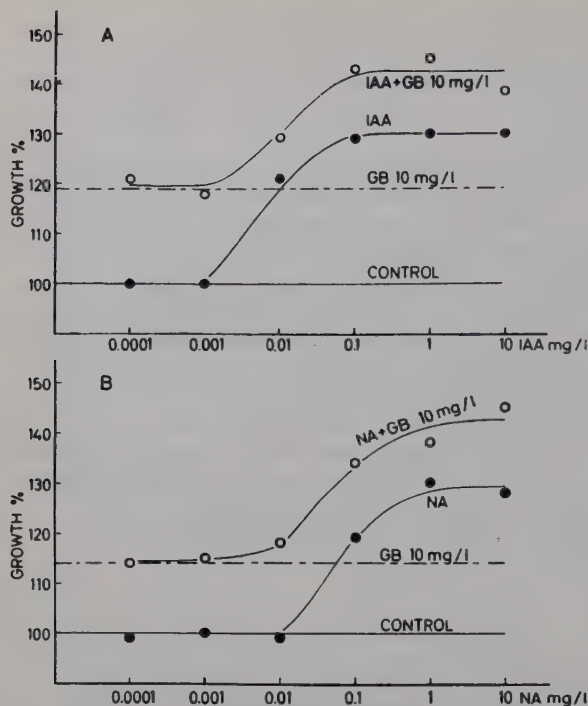


Figure 1. Interaction between GB and auxin within non-effective and effective concentration range of auxin in the elongation of pea stem section. Growth is plotted as percentage of length of control. A. IAA as auxin. B. NAA as auxin.

completely overcome by 0.5 and 1 per cent GB. When GB was 0.1 per cent, the promoting effect of GB was completely suppressed by the presence of 0.1 per cent of various auxin substances, not only NAA but also IAA, 2,4-D and *d*-1,4-dihydronaphthoic acid-(1).

When the cut surface was treated with 0.1 per cent NAA, callus and adventitious root were formed there. But when 0.5 and 1 per cent GB was mixed with NAA, there were little callus formation and no root formation.

Table 1. Effect of GB on IAA-induced growth inhibition of cucumber seedling.

Concentration of GB mg./l.	Concentration of IAA mg./l.							
	0.0		0.05		0.5		5.0	
	R.	S.	R.	S.	R.	S.	R.	S.
0	100 ¹	100 ²	93 ¹	98 ²	54 ¹	64 ²	28 ¹	62 ²
50	103 ¹	125 ²	81 ¹	121 ²	54 ¹	110 ²	28 ¹	100 ²
100	105 ¹	139 ²	90 ¹	131 ²	52 ¹	100 ²	30 ¹	111 ²

In Tables 1—4, the abbreviations, R. and S., stand for root and shoot, respectively.

¹ In each set, differences among the values are not significant at 5 per cent level.

² In each set, differences among the values are significant at 5 per cent level.

Table 2. *Effect of GB on NAA-induced growth inhibition of cucumber seedling.*

Concentration of GB mg./l.	Concentration of NAA mg./l.							
	0.0		0.01		0.1		0.5	
	R.	S.	R.	S.	R.	S.	R.	S.
0	100 ¹	100 ²	73 ¹	100 ²	41 ¹	82 ²	21 ¹	52 ²
50	96 ¹	125 ²	67 ¹	121 ²	40 ¹	105 ²	26 ¹	65 ²
100	88 ¹	159 ²	61 ¹	134 ²	34 ¹	143 ²	26 ¹	100 ²

¹ In each set, differences among the values are not significant at 5 per cent level.² In each set, differences among the values are significant at 5 per cent level.Table 3. *Effect of GB on coumarin-induced growth inhibition of cucumber seedling.*

Concentration of GB mg./l.	Concentration of CM mg./l.							
	0.0		1		20		30	
	R.	S.	R.	S.	R.	S.	R.	S.
0	100 ¹	100 ²	100 ¹	105 ²	60 ¹	62 ²	48 ¹	54 ²
50	100 ¹	135 ²	105 ¹	124 ²	55 ¹	87 ²	48 ¹	73 ²
100	100 ¹	135 ²	99 ¹	147 ²	53 ¹	101 ²	—	—

¹ In each set, differences among the values are not significant at 5 per cent level.² In each set, differences among the values are significant at 5 per cent level.Table 4. *Effect of GB on MH-induced growth inhibition of cucumber seedling.*

Concentration of GB mg./l.	Concentration of MH mg./l.					
	0.0		100		500	
	R.	S.	R.	S.	R.	S.
0	100 ¹	100 ²	78 ¹	70 ²	68 ¹	53 ²
50	106 ¹	124 ²	75 ¹	99 ²	60 ¹	62 ²
100	107 ¹	154 ²	72 ¹	109 ²	56 ¹	72 ²

¹ In each set, differences among the values are not significant at 5 per cent level.² In each set, differences among the values are significant at 5 per cent level.Table 5. *Effect of GB on bud inhibition induced by NAA.*

Compound %		Length of lateral bud as per cent of control
NAA	GB	
0	0	100
0	0.1	224
0.1	0	7
0.1	0.1	7
0.1	0.5	224
0.1	1.0	224

Table 6. *Effect of GB on root-formation induced by IAA.*

Compound IAA	Mg./l. GB	Number of roots per ten plants
0	0	83
0	25.0	29
1.0	0	98
1.0	25.0	49
5.0	0	143
5.0	25.0	60

Effect of GB on auxin-induced root formation. In the above-mentioned experiment, GB acted as an antagonist to auxin in root formation. This effect was studied again using pea cuttings. The results are shown in Table 6. GB itself inhibits root formation in peas as reported by Brian *et al.* (1). And the stimulatory effect of IAA is reduced by GB.

Discussion

The growth of pea stem section was not affected by 0.001 mg./l. of IAA, but was promoted partly by 0.01 and fully by 0.1 mg./l. of IAA (Figure 1 A). If GB were auxin, this part of the concentration-growth curve should be shifted to the left when GB was mixed with IAA. But the growth promotion by GB was simply superimposed on the growth effect of IAA. The same was observed also with NAA (Figure 1 B).

If GB were auxin, it should intensify the growth inhibition by supraoptimal concentrations of auxin by raising the auxin level. But it promoted the elongation of shoot which was inhibited by auxin. It did not either help auxin in inhibiting the root growth (Tables 1 and 2).

GB differs from auxin in that it does not affect the root growth. This nature of GB appeared also when it was mixed with CM and MH. On the other hand, the effect of GB promoting the shoot growth was still apparent when it was combined with these inhibitors.

GB promotes rather than inhibits the growth of lateral bud as does auxin. When mixed with auxin, GB increased the bud growth so much as to cancel the inhibition by auxin (Table 5).

GB also differs from auxin in inhibiting the root formation. And the inhibition works even when the root formation is stimulated by auxin (Table 6).

All the experimental results mentioned above suggested that the action of GB is differentiated from that of the known auxin substances.

Summary

In order to demonstrate the difference between actions of gibberellin and auxin, effect of the mixture of the two was observed.

1. In the concentration ranges of indole-3-acetic acid and naphthalene-acetic acid where shoot growth was, and was not promoted, gibberellin acted additively.

2. In the higher concentration range of indole-3-acetic acid and naphthalene-acetic acid where shoot growth was inhibited, gibberellin did not act additively, but reversed the inhibition.

3. The inhibition of root growth by indole-3-acetic acid and naphthalene-acetic acid was not reversed, nor intensified, by gibberellin.

4. The inhibition of root growth by coumarin and maleic hydrazide was not reversed by gibberellin, although the inhibition of shoot growth by the former was reversed by the latter.

5. Gibberellin inhibited the root formation, and counteracted the stimulative effect of auxin on the root formation.

6. It was concluded that in either shoot or root, gibberellin acts on some point of growth mechanism different from that on which the known auxin acts.

In conclusion, the author wishes to thank Professor Joji Ashida for his guidance, and Dr. Bernard O. Phinney, Associate Professor, University of California, for giving the author valuable advice.

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Studies on the Germination of Date-Palm Seeds.

The Effect of Sodium Chloride

By

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Introduction

Date-palms *Phoenix dactylifera* L. are very common in Iraq and particularly in the middle and the southern regions. One of the most common varieties in the middle of Iraq is the *Zahedi*.

T. H. Kearney in 1911 (c.f. Harris, 3) listed date palm among other crops which succeed in growing in saline soils. He reported that date palm can grow in very strong saline soils which had concentrations of 1.0 to 1.5 per cent.

In the fields around Baghdad the date palm lives in saline soils with 6 per cent total soluble salts and 0.32 per cent chlorides. The present paper deals with the effect of temperature and of sodium chloride or sucrose in varying solutions on the germination of date palm seeds.

Material and Methods

Phoenix dactylifera L. var. *Zahedi* fruits were collected from a date garden near Baghdad. Fresh fruits were collected in September and kept in a cool dry place. Thirty or forty seeds were used per lot. The seeds were germinated on moist cotton in Petri dishes of 14 cm. diameter. In each Petri dish 100 ml. of water, sodium chloride, or sugar solution was used to moisten the cotton. The Petri dishes were incubated at the following temperature series; 8°, 18°, 25°, 27°, 37°, and 45°C. All

experiments on the effect of sodium chloride or sugar were made at the optimal temperature of 25°C. Different concentrations of sodium chloride (c.p.) were used as media for the germination of seeds. Sugar solutions were also used for comparison. Test seeds were surface sterilised with 0.1 per cent HgCl_2 solution for 10 minutes and then washed with sterilized water.

Experimental Results

1. *Effect of Temperature on the Germination of Date Palm Seeds.* Preliminary tests were made by germinating Phoenix seeds in sawdust at room temperature and it was found that the germination was fastest during the months of May and November. Using a range of temperatures it was found (Table 1) that the optimal temperature is 25°C to 27°C. No germination was obtained at 8°C nor at 45°C. Seeds kept at 8°C for four months did not germinate. Seeds at 45°C turned dark brown and the embryo was probably killed. By alternating the day temperature of 27°C and a night temperature of 8°C, 85 per cent germination was obtained by the 19th day. At 18°C 52 per cent germination was obtained by the 19th day, and only 5 per cent germination at 37°C.

2. *Germination of Date Palm Seeds in Sodium Chloride Solutions.* Sodium chloride solutions of different concentrations were used to see how much salinity the date palm seeds could tolerate and at which concentration inhibition occurs. It was found that NaCl solutions suppressed germination during the early stages of germination. Figure 1 shows clearly that 0.5 per cent NaCl solution inhibited germination during the first 20 days, but final percentage of germination was equal to that of the control, i.e. distilled water. It reached 100 per cent after 35 days. Similar results were obtained with 1 per cent NaCl, but with a higher rate of inhibition, at the 20th day there was 50 per

Table 1. *Effect of temperature on the germination of Zahedi date palm seeds.* Forty seeds were used for each temperature.

Temperature °C	% germination		
	13 days	16 days	19 days
8	0	0	0
18	0	0	52.5
25	82	96.6	100
27	80	100	100
37	0	2.5	5
45	0	0	0
27 + 8 ¹	0	62.5	85

¹ 27°C day temperature for 10 hrs. daily and 8°C night temperature for 14 hrs. daily.

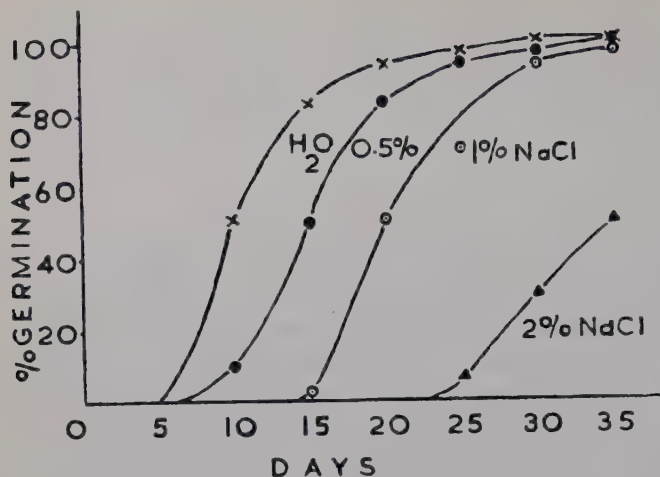


Figure 1. Germination of date-palm seeds in different concentrations of sodium chloride at 25°C.

cent inhibition then the inhibition decreased with time reaching the maximum germination at the 35th day. Seeds in 2 per cent NaCl solution revealed no germination before the 25th day, the maximum germination never exceed 50 per cent (Figure 1). Figure 1 also shows that with every 0.5 per cent increase in NaCl there is about 5 days delay in germination.

In order to find the maximum concentration of NaCl which date palm seeds could tolerate the following experiment was made. Eleven lots of Petri dishes with 30 seeds each and 100 ml. of NaCl solutions ranged from 0.1 to 2.5 per cent. The results are illustrated in Figure 2. The increase in the NaCl concentration decreases germination. It was of interest to note that germination was obtained even with solutions as high as 2 per cent NaCl. There was no germination at 2.5 per cent or higher concentrations of NaCl. Sodium chloride in concentrations of 0.2 up to 0.8 per cent had practically no inhibitory effect on germination up to the 25th day. Concentrations of 1 per cent or higher greatly inhibited germination. Figure 2 shows the percentage of germination of date palm seeds at the 10th, 15th, and the 25th day. Readings at later periods; 35, 45, 60, and 80 days were taken, but there was no significant increase in percentage of germination after the 25th day. Advances in morphological development were observed with additional time allotment.

The osmotic concentration of NaCl solutions ranged from 0.81 atm. (0.1 per cent NaCl) to 19.15 atm. (2.5 per cent NaCl), (4). It seems that the date palm embryo can tolerate up to 15.36 atm. osmotic pressure at 25°C. Seeds germinated at this high concentration of NaCl solution did not develop normal seedlings, i.e. the growth of seedlings was stunted. It was noticed

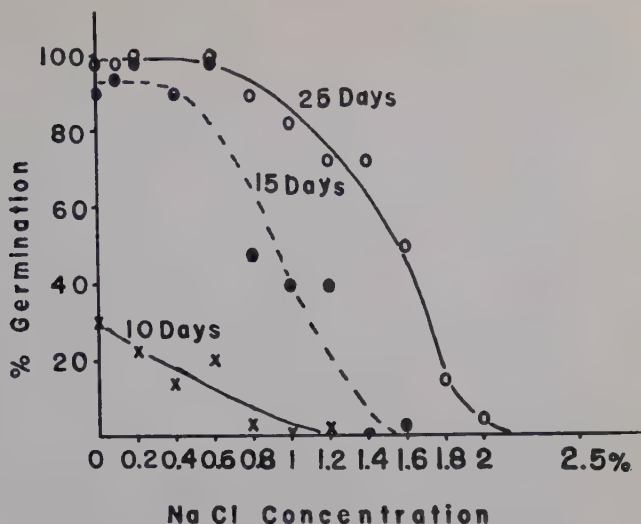


Figure 2. Relation between NaCl concentration and germination of date-palm seeds. Forty seeds per lot were used at 25°C.

that germination was not very much affected by 9.27 atm. osmotic pressure of NaCl solution. Solutions of NaCl with osmotic pressure higher than 9.27 atm. inhibited germination when measurements were made on the 25th day from soaking at 25° C. The inhibition of seed germination increased with the increase in the osmotic pressure of the external solution.

3. *Morphological Changes During Germination.* To find the development of date seedlings during the first 35 days, seeds were germinated in distilled water at 25°C and observations were made daily. The first external morphological changes were observed at the 10th day (Table 2). The radicle appeared by the 10th day and continued to elongate till the 14th day. The cotyledonary sheath appeared after the 22nd day. The first primary leaf is seen after the 34th day and the lateral roots are formed at the 40th day.

4. *Effect of Sugar on Date Seeds Germination.* Different concentrations of sucrose solutions were used as media for germination of date palm seeds

Table 2. Morphological changes of date-palm seeds germinated in distilled water at 25°C.

Time from the beginning of experiment	Changes during germination
1—10 days	No external changes
10—14 "	Appearance of the radicle
14—22 "	Elongation of radicle
22—26 "	Appearance of cotyledonary sheath
26—34 "	Elongation of cotyledonary sheath
34—38 "	Appearance of first primary leaf
38—44 "	Appearance of lateral roots

Table 3. *Percentage germination of date palm seeds in sucrose solutions at 25°C.*

% sucrose	Osmotic pressure	12 days	15 days
0	0	82.5	92.5
0.5	0.337	77.5	95.0
1.0	0.675	60.0	92.5
1.5	1.022	62.5	95.0
2.0	1.349	42.5	80.0
2.5	1.687	30.0	57.5
3.0	2.024	27.5	57.5
3.5	2.364	30.0	42.5
4.0	2.698	12.5	45.0
4.5	3.036	10.0	27.5
5.0	3.374	7.5	20.0

in comparison with that of sodium chloride. The solutions ranged from 0 to 5 per cent sucrose. The results are tabulated in Table 4, which shows that seed germination was inhibited greatly when sucrose concentration was increased. More than 50 per cent inhibition was observed with only 3.5 per cent sucrose solution, and 20 per cent germination was obtained with 5 per cent sucrose, at the 15th day. If seed germination in sucrose solution is compared with that of NaCl solution of the same osmotic pressure, we find that seed germination is very much inhibited in the case of sugar. To compare the germination in 3 per cent sucrose solution which has 2.02 atm. osmotic pressure with a similar osmotic pressure of NaCl solution, *i.e.* 1.55 to 3.07 atm (0.2—0.4 per cent NaCl) we find only 57.5 per cent germination in the former case while 92.5 per cent germination in the later case. The inhibition related to osmotic pressure is greater by far in the case of sugar solution.

Discussion and Conclusions

Brown and Bahgat (2) worked with *Hayani* variety of date-palm seeds and reported that dehydration of date seeds at 40° to 50°C for 30 to 120 minutes germinated at the shortest period when the temperature for germination was 35°C. No other work was done on the optimal temperature for the germination of date-palm seeds. Experiments reported in this paper using *Zahedi* date seeds showed that the optimal temperature is 25° to 27° C. Germination at this temperature occurs within 12 days.

Uhvits (5) found that the germination of alfalfa seeds was practically inhibited at osmotic pressures of 12 to 15 atm. of NaCl. He observed that the mortality of seedlings was very high in sand cultures containing NaCl solutions of 7 to 9 atm. osmotic pressure. In date-palm seeds germinated in NaCl

solution of 15.36 atm. osmotic pressure (2 per cent NaCl) gave 50 per cent germination after 35 days. Solutions of NaCl with osmotic pressure higher than 15.36 atm. completely inhibited germination. Ayers' work (1) with sugar beet can be compared with the results of Figure 1 of this paper. In date-palm seeds we have seen that 0.5 per cent NaCl solution gave 50 per cent germination at the 15th day. In sugar beet Ayers obtained only 20 per cent germination with 0.1 per cent NaCl at the 15th day. It is clear that date seeds can tolerate much more salt than sugar beet.

Sucrose has an inhibitory effect on seed germination. Solution of 5.01 per cent sucrose which has 3.37 atm. gave 20 per cent germination by the 15th day. Seeds germinated in lower concentrations of sucrose, *i.e.* 0.5 to 1.5 per cent sucrose produced no inhibition. The interesting observation with the germination of date-palm seeds is that sucrose solution with a particular osmotic pressure is much more inhibitory than sodium chloride of the same osmotic pressure. Seeds germinated in NaCl solution of 3.7 atm. had a much higher rate of germination than those germinated in a sugar solution of 3.04 atm. with a maximum of 27.5 per cent at the same temperature. This difference in the rate of germination may be explained in terms of interference of sucrose with carbohydrate metabolism of the germinating seed. Sucrose solution entering through the micropyle may delay the carbohydrate hydrolysis of the seed and hence delay the growth of the embryo.

Summary

1. Date-palm, *Phoenix dactylifera* L. var. *Zahedi*, seeds were collected from Baghdad were used in this investigation. Seeds were germinated in water or solutions in Petri dishes.

2. The optimum temperature for germination of date seeds was 25—27°C.

3. Low concentrations of NaCl solutions, 0.1 to 1 per cent NaCl, showed some inhibition in the early stages of germination, and concentrations of 2 per cent NaCl or higher showed complete inhibition.

4. Morphological changes are obvious 10 days after the addition of water, these morphological changes start with the emergence of radicle, cotyledonary sheath, the first leaf, and the appearance of lateral roots which are seen by the 44th day.

5. Sugar solutions higher than 1.5 per cent showed inhibition in seed germination. Sugar solutions with osmotic pressure similar to those of NaCl solutions have much more inhibition to seed germination.

The author wishes to thank Professor J. C. Russel of the College of Agriculture, Abu-Ghraib, for valuable suggestions.

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Component Sugars of Mycelium and of Polysaccharide Material Produced in Liquid Culture by *Verticillium albo-atrum*

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Introduction

Polysaccharides have been detected in the culture filtrate when *Verticillium albo-atrum* Reinke et Berth. is grown on liquid media. For some time it was believed that such materials were toxins partially responsible for the symptoms exhibited by diseased plants.

Bewley (1), as early as 1922, found that culture filtrates from *V. albo-atrum* contained materials that caused wilting of tomato cuttings. These materials were heat labile and could be precipitated from solution with ethanol. Porter and Green (6) reported that the toxic fraction was a complex polysaccharide. They concluded the polysaccharide was of variable molecular size as there appeared to be at least two fractions. One of the fractions passed through a Seitz filter while the other did not.

Later Green (3) reported that wilting of tomato cuttings was due to proteinaceous materials in the culture medium. Precipitation of the materials from solution with 95 per cent ethanol destroyed the ability to cause wilting of the cuttings. In addition to the protein fraction, a polysaccharide was also present in the medium. Green found that the polysaccharide was responsible for vascular discoloration, but that it was not responsible for wilting. Experiments indicated that the polysaccharide was a polymer of hexose sugars and that glucose was the principal, and possibly the only, constituent sugar.

Recently Scheffer *et al.* (7) found that culture filtrates of *V. albo-atrum* caused wilting and vascular browning of tomato cuttings. Heating the filtrates eliminated the vascular browning, but the cuttings still wilted. They ascribed this wilting to non-specific material, such as polysaccharides.

While there seems to be little doubt that *Verticillium albo-atrum* can produce a polysaccharide in culture, very little is known about the nature of this material. In conjunction with research carried out with *Verticillium* wilt of potatoes, it was of interest to characterize more fully the polysaccharide materials produced in a liquid medium by *V. albo-atrum*.

Materials and Methods

All isolates of *V. albo-atrum* were originally obtained from diseased potato plants by Dr. James Guthrie of the Aberdeen Branch Experiment Station, Aberdeen, Idaho.

Unless otherwise noted, the fungus was grown on a synthetic medium of the following composition: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; KCl, 0.5 g.; KH_2PO_4 , 1.0 g.; NaNO_3 , 3.0 g.; 0.5 ml. of an $\text{Fe}(\text{NO}_3)_3$ solution; 0.5 ml. of a minor salts solution; sucrose, 30 g.; and distilled water added to make a final volume of 1 liter. The final concentration of Fe was 0.2 mg./l. while the final concentration of Mo and Mn was 0.02 mg./l. and Cu and Zn was 0.1 mg./l. Mn, Cu, and Zn were added as the sulfates and Mo as sodium molybdate. The medium was adjusted to pH 4.5, dispensed 25 ml./125 ml. Erlenmeyer flask, plugged with cotton and autoclaved 15 minutes at 15 lbs pressure. Flasks were inoculated with a loop of mycelial suspension and incubated at room temperature (21—25°C.).

The sugars present in the various fractions were identified by paper chromatography following acid hydrolysis (HCl or H_2SO_4) of the fractions. Hydrolyses with 1 N HCl were carried out for 2 hours in sealed tubes in a boiling water bath. Each tube contained 10—20 mg. of material per ml. of 1 N HCl . In most cases 5—30 microliters of the hydrolyzates could be chromatographed directly without removal of the chloride. With very dilute hydrolyzates, the Cl was removed by adding silver oxide until the liquid was slightly acid (pH 4—5). These solutions were concentrated *in vacuo* prior to chromatography.

Hydrolyses with 1 N or 0.25 N H_2SO_4 were made by refluxing for 2 hours. Usually H_2SO_4 hydrolysis resulted in excess frothing. Following hydrolysis the sulfate was removed by adding BaCO_3 until the aqueous supernatant was slightly acid (pH 4—5). These solutions were then concentrated *in vacuo* prior to chromatography.

The hydrolyzates were chromatographed by one dimensional descending technique on Whatman No. 1 filter paper. Irrigants used included the organic phase of phenol saturated with water, butanol-acetic acid-water (4/1/5, v/v/v) and the freshly prepared one phase mixture of ethyl acetate-pyridine-water (8/2/1, v/v/v). Unknown materials were identified by comparing with known sugars on the same chromatogram following the location of the spots with various spray reagents. These spray reagents included 3,5-dinitrosalicylic acid, aniline hydrogen phthalate and *p*-anisidine-HCl (2). The latter two sprays yield characteristic colors with various sugars that further aid in identification of sugars in the unknown.

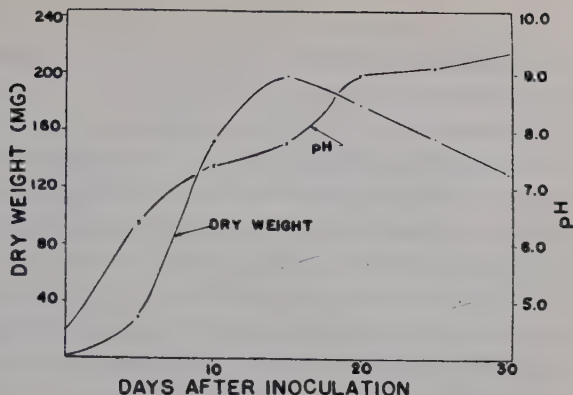


Figure 1. Growth curve and concurrent pH changes for *Verticillium albo-atrum* grown in a sucrose salts medium.

Results and Discussion

The growth of the fungus in the synthetic medium and the changes in pH of the culture medium in a typical experiment are shown in Figure 1. The mycelium was collected on sintered glass crucibles, washed with distilled water to remove residual salts and dried at 90°C. The mycelial mat attained maximum weight in approximately 15 days and then began to lyse. As a result of the fungus growth, the pH of the medium increased until the medium became quite alkaline. Different isolates of the same organism will vary somewhat in the amounts of dry weight produced and in the rapidity and extent of lysis.

Green (3) found that the production of polysaccharides increased after the cultures were approximately 20 days old. After this time the concentration of polysaccharides increased steadily as the culture lysed. Qualitative experiments in this study were in general agreement with those of Green. During the first 5—15 days after inoculation, the addition of alcohol to the culture medium resulted in small quantities of light flocculent precipitates. After approximately 15 days the amount of precipitate increased. Paper chromatographic determinations showed no detectable quantities of sucrose, glucose, or fructose in the culture medium after approximately 15 days growth. As the culture became older, more alcohol was necessary to precipitate out the materials from the culture filtrates. Thus, the addition of two volumes of 95 per cent ethanol to filtrates from 15-day old cultures caused a precipitate to form immediately. The addition of three volumes of ethanol to a filtrate from 33-day old cultures resulted in a small amount of precipitate and a cloudy supernatant that remained stable for at month at 40°C. Adding one more volume of ethanol resulted in a finely divided precipitate

and a clear supernatant. The increasing solubility in alcohol might indicate that the molecular size of materials in solution was decreasing.

A preliminary experiment was made to determine the constituent sugars of polysaccharides present in culture medium. Two volumes of 95 per cent ethanol were added to 200 ml of culture filtrate from a 22-day old culture and the mixture set aside at 4°C. The precipitate was collected by filtration and the wet precipitate washed with acetone. Washing with acetone (or with ethanol and finally diethyl ether) resulted in a white to light gray product. Failure to use acetone yielded dark colored precipitates that adhered to the filter paper. Thirty mg of material was obtained, hydrolyzed and chromatographed. Substances chromatographically identical with glucose, mannose, galactose, and arabinose were found.

In addition to polysaccharides present in solution, the precipitate in the above experiment may have contained particulate material such as spores and mycelial fragments. To overcome this difficulty, further experiments were made in which the culture medium was Seitz-filtered prior to ethanol precipitation. Twenty-nine mg of material was obtained by alcohol precipitation of 200 ml of Seitz-filtered medium from a 23-day old culture. Similar treatment of the filtrate from a 30-day old culture resulted in the recovery of 115 mg of material. These yields are not quantitative due to some loss in manipulation; nevertheless, the results demonstrate the increase of material in solution as the culture ages. Comparable results were obtained with another isolated of *V. albo-atrum*.

Following hydrolysis, chromatograms of the Seitz-filtered alcohol-insoluble material showed spots corresponding to known glucose, galactose, mannose, and arabinose. On the basis of spot size and intensity, glucose and galactose were present in greater concentration than mannose and arabinose. Semi-micro-Kjeldahl determinations showed a protein content ($\% N \times 6.25$) of 10 to 16 per cent in various preparations of this material.

Experiments were made to determine the sugar composition and protein content of the polysaccharides present in the mycelium. Mycelial mats were chopped in a Waring Blendor with sufficient ethanol to yield a final concentration of about 80 per cent ethanol. The insoluble residue was filtered off and further extracted in a Soxhlet extractor. Three samples of this alcohol insoluble material collected in different experiments contained from 30 to 35 per cent protein.

Hydrolyzates of the alcohol-insoluble portion of the mycelial mats produced spots chromatographically indistinguishable from known glucose, galactose, mannose, arabinose, and ribose. Judging from spot size and intensity, glucose appeared to be the predominant sugar. Galactose and arabinose occurred in lesser and about equal concentration while ribose and mannose

were present in low concentration. Uronic acids, normally found in polysaccharides of higher plants, were not detected in the mycelium. Qualitatively, these five sugars were found in mycelium during all stages of growth. As might be expected, these same sugars were present in the mycelium when other materials, such as glycerol, glucose, and *n*-acetyl glucosamine, served as carbon sources in the medium. Qualitatively there were no differences in the component sugars of mycelium of three isolates of the fungus.

It has been shown by chromatographic analyses that culture filtrates contain unknown ketose materials (5). It seemed possible that the mycelium might also contain ketose materials, such as fructose, in a combined state. As hydrolysis with 1 N acid might have destroyed sugars such as fructose, hydrolyses of the mycelium were repeated with 0.25 N H₂SO₄. Chromatographic examination of these weak-acid hydrolyzates did not show detectable quantities of any ketose sugar.

In order to investigate further the solubility of the polysaccharide material in the mycelium, surface mats from 22-day old cultures were extracted with water instead of ethanol. The mixture was filtered on a Buchner funnel and the residue washed with distilled water, and finally with acetone. The aqueous filtrates were combined, two volumes of 95 per cent ethanol added, and the mixture allowed to stand at 4°C. for several days. The precipitate was collected by filtration and washed with acetone.

Chromatographic examination of hydrolyzates of the water-insoluble residue showed that glucose, galactose, mannose, arabinose and ribose were present. Judging from spot size and intensity with spray reagents, the insoluble residue contained predominantly glucose, lesser amounts of galactose and mannose and very small quantities of arabinose and ribose. Spots for the two pentoses were seen only when large quantities of hydrolyzates were chromatographed. This often resulted in somewhat streaked chromatograms. Nevertheless, distinct spots yielding pink to brown red colors with the *p*-anisidine and aniline phthalate spray reagents were distinguishable.

Hydrolyzates of the water-soluble, alcohol-insoluble fraction of the mycelium also gave spots indistinguishable from known mannose, glucose, galactose, arabinose, and ribose. Galactose and glucose were prominent while the other three occurred in lesser but easily detectable amounts. It would thus seem that ribose and arabinose were almost completely removed from the older mycelium by water extraction. As the ribose appears to be quite easily removed from the mycelium with water extraction, it is surprising that ribose was not found in the culture filtrate.

These experiments show that polysaccharide material appears in quantity in the culture filtrate only after the mycelial mat had begun to lyse. Further, the polysaccharide material in the culture filtrate does not appear to be

unique as the component sugars in this fraction were almost identical with those occurring in the mycelium. Any wilting of plant cuttings caused by such materials would probably be due to non-specific wilting similar to that caused by other large molecules (4).

Summary

The experiments in the present study reaffirm the occurrence of polysaccharide and protein material in the culture medium of *V. albo-atrum*. These material appeared in quantity in the medium only after the carbon source was depleted and the mycelial mat had begun to lyse.

The alcohol-insoluble material of Seitz-filtered culture medium yielded glucose, galactose, mannose, and arabinose when hydrolyzed. As the culture aged, more alcohol was required to bring about precipitation of materials in the culture filtrate. In addition to the polysaccharide material, this insoluble fraction contained 10 to 16 per cent protein.

Five sugars, glucose, galactose, mannose, arabinose, and traces of ribose, were present in the mycelium of *V. albo-atrum*. The same sugars were present in material from a water-soluble extract of mycelial mats that have begun to lyse. The mycelium contained 30 to 35 per cent protein.

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Response of Pea Stem Sections to Indoleacetic Acid, Gibberellic Acid, and Sucrose as Affected by Length and Distance from Apex

By

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Introduction

In the original description of the pea stem section straight growth test, Galston and Hand (8) indicated that such sections show increased growth in response to sucrose. Christiansen and Thimann (4), on the other hand, presented data showing that these sections can attain maximum growth without added sucrose. The latter obtained a slight sucrose response by using short sections, but they were unable to obtain as strong a response as did Galston and Hand. Leopold (12) has attempted to explain this difference in terms of light action. Audus (1) has suggested that the time factor is of importance. While working independently in the same laboratory we have likewise obtained discrepant sucrose responses as well as different values for the indoleacetic acid (IAA) concentration producing maximum elongation, although all tests were run under apparently identical conditions. The following experiments, undertaken to resolve these discrepancies, show that section length and distance from the apex are of great importance in determining the responses to sucrose, IAA, and gibberellic acid (GA).

Materials and Methods

Seeds of *Pisum sativum* L., var. Alaska, were obtained from Asgrow, Inc., of New Haven, Connecticut, sown in vermiculite (Mica-Gro Type B, supplied by Platt Seed

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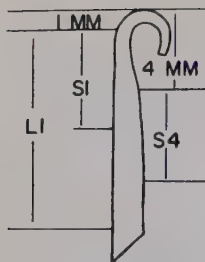


Figure 1. *Debudded apex of dark-grown seven day old etiolated pea plant showing location of S1, L1, and S4 sections.*

Co., Branford, Connecticut, and thoroughly washed in running water) and allowed to develop for 7 days at $26 \pm 1^\circ \text{C}$.

Experimental plants were either "dark-grown" or "red-grown". The former were kept in darkness except for exposure to photosynthetically and morphogenetically inactive dim green light at time of handling; these were selected for recurved apical crooks. Red-grown plants, obtained by exposure to red light (two 15-watt red fluorescent tubes at a distance of 30 cm.) for 20 minute periods at 3-hour intervals during the 18 hours before harvesting, were selected for 90° apical crooks. All sections were taken from plants having third internodes between 15 and 40 mm. in length. Sections were cut on a van der Weij "guillotine" (8) and then randomized in buffer. Lots of 10 sections were weighed and placed on circles of Whatman no. 1 filter paper in 10-cm. petri dishes containing 8 ml. of medium. Each dish was divided into 2 halves by a microscope slide under the filter paper; thus each experimental treatment contained 2 lots of 10 sections. The basal medium consisted of $0.02 M \text{KH}_2\text{PO}_4\text{—Na}_2\text{HPO}_4$ buffer (pH 6.1); 2 per cent sucrose was also present except in the determination of sucrose requirement. The sections were measured and reweighed in lots of 10 after 20 hours of growth in darkness.

The following abbreviations will be employed: "S" sections are 5 mm. in initial length; "L" sections are 10 mm. long. "S1" sections are 5 mm. sections taken 1 mm. below the apical crook (Figure 1), "S4" sections are taken 4 mm. below the crook, and so forth.

Results

Response to Sucrose

Growth of S1, S4, L1 and L4 sections of dark-grown peas was tested in the presence and absence of 2 per cent sucrose. $10^{-7} M$ IAA (suboptimal for all sections except S1, see below) was added since elongation of S4 and L4 sections in buffer alone is so small that any differences observed might not be meaningful. The data in Figure 2 show that S1 sections responded strongly to sucrose and L4 sections were unaffected, while the others showed an intermediate response. All 4 types of red-grown sections responded positively to sucrose, the effect being more pronounced in the S1 and S4 sections.

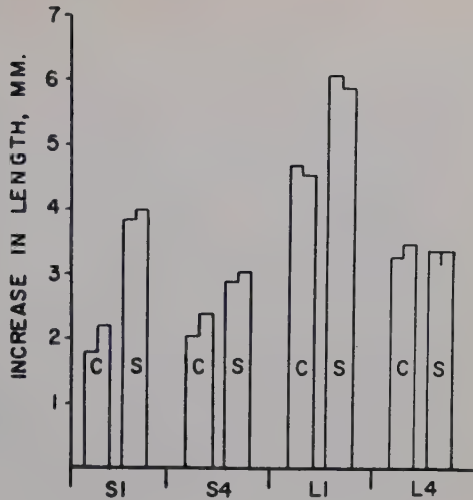


Figure 2. *Effect of 2 per cent sucrose on elongation of S1, S4, L1, and L4 sections of dark-grown pea stems. Control medium: 0.02 M phosphate buffer (pH 6.1), 10^{-7} M IAA. Divisions of bars represent replicate lots of 10 sections. C: control; S: sucrose.*

Response to IAA

IAA caused maximum fresh weight increase of S1, S4, L1, and L4 sections of dark-grown peas at a concentration of 10^{-5} M with little decline at 10^{-4} M, whereas 10^{-4} M was optimal or suboptimal for red-grown sections. Figure 3 shows, however, that distance from apex strongly affects the elongation response of dark-grown sections to IAA. S1 sections showed a maximum response at 10^{-7} M, while concentrations greater than 10^{-6} M caused an inhibition of elongation. The absolute response of such sections to optimal IAA was usually extremely small. S4, L1, and L4 sections showed a much greater response to IAA, and the optimal concentration was 10^{-6} M. None of these

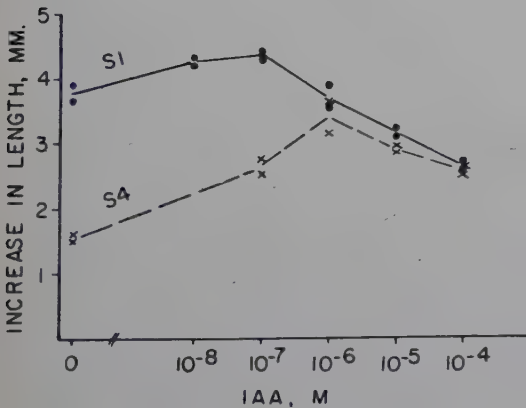


Figure 3. *Effect of distance from apex on elongation response of dark-grown pea stem sections to IAA. IAA concentration curves for S1 and S4 sections. Control medium: 0.02 M phosphate buffer (pH 6.1) plus 2 per cent sucrose. Each point represents the average of a lot of 10 sections.*

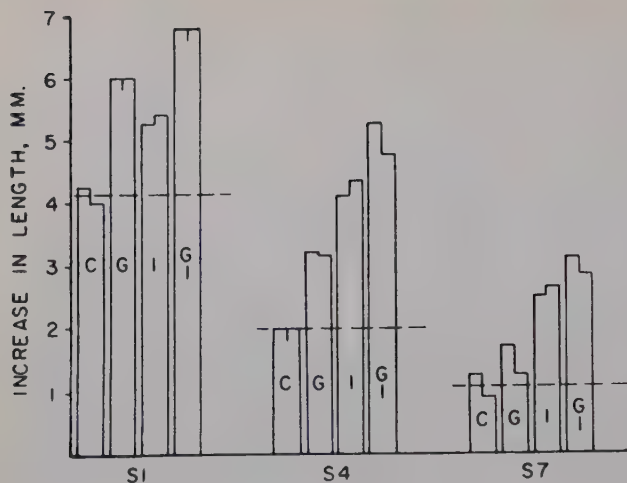


Figure 4. *Effect of distance from apex on elongation response of dark-grown pea stem sections S1, S4, and S7 to GA (10^{-5} M) and IAA. IAA in each case at optimal concentration for elongation, i.e., 10^{-7} M for S1, 10^{-6} M for S4 and S7 sections. Control medium: 0.02 M phosphate buffer (pH 6.1) plus 2 per cent sucrose. Divisions of bars represent replicate lots of 10 sections. C: control; G: GA; I: IAA.*

were inhibited in elongation by concentrations up to 10^{-4} M. Red-grown sections of all 4 types showed maximal elongation response at 10^{-4} M IAA; this confirms the results of Galston and Baker (6), who found that pretreatment of the plants with red light increased the concentration of IAA required to obtain maximum elongation.

Response to GA

GA was used at a concentration of 10^{-5} M since it is known (3, 9, and our own preliminary results) that the response of pea stem sections to GA is relatively constant over a broad concentration range. Figure 4 shows data comparing the elongation responses of dark-grown S1, S4, and S7 sections to GA, to IAA (at concentrations optimal for each type of section), and to the two combined. As the distance from the apex increased, the endogenous growth decreased. Similarly, the response to GA was sharply lowered. The response to optimal IAA was greater in the S4 and S7 sections, and the relative response to IAA as compared with GA increased steadily with the distance from the apex. There was an indication of a slight synergism between GA and IAA on S4 sections in one experiment. Fresh weight increase responses were similar to those for elongation. As in all our experiments, the effect of increased section length was to increase growth at a given distance from the apex. In red-grown sections the effect of increased distance from the apex was, again, to decrease the endogenous and GA-induced growth. Also, red light pretreatment itself lowered the endogenous and GA-induced growth.

Discussion

The disagreement between the data of Galston and Hand and of Christiansen and Thimann on sucrose requirements is now easily explained, since the former were based on 5 mm. sections relatively close to the apex and the latter on 20 mm. sections cut at an unspecified distance from the apex. The failure of Christiansen and Thimann to obtain as great a sucrose response even when using 5 mm. sections probably resulted from cutting sections at a greater distance from the apex.

In seeking an explanation for a relatively minor disagreement, we have found several interesting effects. The effects of increasing distance from the apex are to reduce the endogenous growth and the response to GA and sucrose and to increase the relative sensitivity to IAA as compared with GA. The effects of increased section length are to reduce the sensitivity to sucrose (presumably because of the presence of greater amounts of stored carbohydrates) and to increase the total elongation at a given distance from the apex. An important property of long sections is that they contain tissues heterogenous in their responses to IAA and GA. It is also evident that the technique of taking serial sections from the same plant, employed by Galston and Hand and others, is inadvisable when working with dark-grown peas. However, most work with pea sections reported to date has involved the use of a red safelight, so that such considerations are not of such great importance as when a green safelight is employed. Sections from red-grown plants show weaker effects of distance from apex. One of us has reported in a lecture (10) that dark-grown sections are insensitive to sucrose while red-grown sections require it. Since the sections compared were essentially similar to our L4 sections, the results do not conflict with those given here. The small effects of gibberellin on elongation of pea stem sections reported by others (3, 9, 11) may be attributed to the cutting of sections at a greater than optimal distance from the apex.

S1 sections have a number of striking properties: their high endogenous growth rate, weak response to IAA, low IAA optimum, and extreme sensitivity to gibberellin. The first three may perhaps be explained in terms of proximity to the site of auxin production and a high endogenous auxin content. On the other hand, this cannot explain the high sensitivity to GA, although high endogenous and GA-induced growth appear to be closely linked. That GA-induced growth is not proportionately enhanced by IAA is seen in Figure 4. When S4 sections are induced by IAA to equal the endogenous growth of S1 sections, their response to added GA is still lower than that of the S4 sections. There is a similar relationship between GA responses of S4 and IAA-induced S7 sections. Brian and Hemming (2) have reported

a synergism between GA and IAA in a different system. While in our system there were indications of a slight synergism, the responses to GA and IAA were in general simply additive. The results of Vlitos and Meudt (13), indicating an interaction between gibberellic acid and a factor(s) in the apex of pea seedlings, invite comparison with our data. Whether our results may be interpreted in terms of interaction with "apex factors," greater sensitivity of immature tissue or of tissue with a high endogenous growth rate, or none of these, remains to be seen.

Our findings bear significantly on assays for both auxins and gibberellins. Smaller sections taken from very young portions of the stem may provide a satisfactory bioassay material for the gibberellins. In using the pea stem section growth test as an assay for auxins, however, it is necessary to take sections from a sufficiently mature portion of the stem to obtain a strong auxin response and avoid interference by gibberellins.

The observation of differing IAA effects on increase in weight and length of sections confirms the findings of de Ropp and Markley (5), Galston *et al.* (7), and others. Particularly noteworthy is the fact that while IAA concentration curves for elongation of S1 and S4 sections (Figure 3) appear qualitatively different, corresponding curves for fresh weight increase run almost perfectly parallel, with S1 merely starting from a higher endogenous level. Such differences must be important in understanding the mechanism of auxin action.

Further investigation of these and other factors contributing to the growth of pea stem sections are in progress.

Summary

(1) The further the sections are from the apex, the less is the endogenous growth and the response to GA and sucrose, and the greater is the relative response to IAA as compared with GA.

(2) Longer sections are less sensitive to sucrose, and they show greater elongation than the corresponding short sections at a given distance from the apex. Longer sections may contain tissues heterogeneous in their response to IAA and GA.

(3) Our results underline the necessity of accurately specifying the type of sections employed in pea section growth tests. Small sections taken very close to the apex may prove to be a useful bioassay material for the gibberellins.

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Metabolic Responses to Auxin III.

The Effects of Auxin on ATP Level as Related to the Auxin Induced Respiration Increase

By

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(Received August 21, 1957)

The remarkable action of auxin in increasing the ratio reduced/oxidized glutathione in isolated plant parts has been described in a previous communication (8). The probable relation of this effect with the auxin induced stimulation of oxygen uptake prompted us to some investigations on the mechanism by which auxin stimulates respiratory activity. The particular point dealt with in this report is whether the effect of auxin on respiration is to be considered as primary, or as consequent to that on growth.

The mechanism by which auxin increases respiration is still obscure. Considerable attention has been given to the possibility of an auxin induced activation of respiratory enzyme systems. Berger and Avery (1) and Teubner and Murneek (12) found a higher activity of several Krebs cycle dehydrogenases in auxin treated tissues. Stimulating effects of growth hormones on dehydrogenase systems in cell free preparations have also been reported (12), (7), (9), but these results appear far from being satisfactorily reproducible. The possibility that specific terminal oxidases are involved in growth has been suggested by Thimann et al. (13), and an increase of catalase and respiratory activities in auxin treated orchid ovaries has been reported by Hsiang (6). An indirect effect of auxin on dehydrogenase systems, consequent to a direct action of auxin on ascorbic acid oxidation has been recently proposed by Marrè and Arrigoni (8).

A completely different, and quite interesting interpretation of the oxygen

uptake activating effect has been recently suggested by Bonner *et al.* (2). These authors basing themselves on the observation that in the artichoke tuber tissue the respiratory activation by auxin does not sum up with that induced by DNP (a reagent uncoupling phosphorylations from oxidations), as well as on other considerations, conclude that the auxin induced increase of oxygen consumption should be considered as a consequence of the effect on growth. Auxin would primarily increase some metabolic work connected with growth (as water uptake), and thus the utilization of high energy phosphate bonds. Consequently phosphate acceptor availability would rise, and oxidative reactions would be accelerated.

Attractive as Bonner's hypothesis is, it seems difficult to conciliate with some observations we made in studying the metabolic effects of auxin. In a research on dehydrogenase behaviour in pea internode segments (dehydrogenase activity being estimated *in vivo* by the tetrazolium method) we observed that the activating effect of auxin and of 2,4-DNP appeared, in most experiments, quite independent from each other, and that in the presence of both factors the activation values were practically equal to the sum of each factor when supplied alone. This seemed to indicate a complete independence of the auxin effect on respiration from the concentration of phosphate acceptors in the cell, and thus a somewhat direct effect of the hormone on oxidative systems. Unfortunately, the coarseness of the technique used and the poor reproducibility of the results made it difficult to draw any definite conclusion from these experiments.

The obvious importance of the problem induced us to try a more direct approach, that is, the determination of the changes of ATP level (or, rather, of the ATP/ADP ratio) in the first period following auxin treatment of isolated plant parts. It seemed reasonable to assume that an increased high energy phosphate utilization could stimulate oxidative reactions only inasmuch as a decrease of ATP, and an increase of ADP were involved: and, conversely, that if an increase of ATP were found, this would mean that auxin induced stimulation of respiratory activity was due to some other cause than the increase of metabolic work connected with growth.

The results here related show that the activating effect of auxin on respiration is accompanied, at least in its initial phase, by a significant increase of the ATP level and, therefore, that it cannot be considered as a mere consequence of the increased metabolic work, but, rather, as due to an auxin induced activation of oxidative reactions. The action of auxin on respiratory and energetic metabolism is thus interpreted as involving the following successive steps: a) activation of respiratory system; b) increased synthesis of high energy bonds; c) increased capacity for metabolic work; each step being a direct consequence of the preceding one.

Methods

One cm. long segments from the apical part of the third internode of etiolated Alaska pea seedlings cultured for 7 days in a thermoregulated darkroom at 25°C, washed and randomized, have been disposed in petri dishes containing distilled water or a 5×10^{-5} M indoleacetic acid solution. At the end of the treatment (darkness, 25°C) the segments were ground in a mortar with 1 vol. of 10 per cent cold perchloric acid and some quartz sand, centrifuged at 3000 r.p.m. and the sediment washed twice with 5 per cent perchloric acid. The washings were added to the main extract, and the acid extract analysed for ATP and ADP labile phosphate by the method of Crane and Lipmann (4) as modified for plant material by Forti (5). According to this procedure the charcoal Norit A is added to the acid extract in two steps, each addition being followed by centrifugation. The sedimented charcoal is resuspended and centrifuged at $2000 \times g$ for 5 min., 0.2 ml. of 95 per cent ethanol being previously layered on the surface to reduce the amount of charcoal that floats. The supernatant is discarded, and the charcoal with the adsorbed adenine containing nucleotides is washed 3 times with distilled water, ethanol being added each time; 1-N HCl is then added to the centrifuge tubes and the tubes disposed for 10 min. in a boiling bath to hydrolyze the labile phosphate of ATP and ADP. The liquid in the tubes is then brought to volume, the charcoal is filtered by suction and the clear filtrate is analysed for phosphate. By this procedure in the pea tissue recovery of initially present ATP is quite satisfactory, and no other easily hydrolyzable phosphate than that of nucleotides is determined: contamination by inorganic phosphate being also extremely limited (from 0.3 to 0.4 per cent). The labile phosphate determined by this procedure will thus be referred to as high energy bonded phosphate of nucleotides, ($\sim P$); though some minor contamination by part of some unknown labile P-containing compounds with different biochemical meaning cannot be excluded.

Specific, reproducible data on the ATP contents in the pea tissue extracts were obtained by combining the procedure for nucleotide labile phosphate with the treatment of aliquots of the extracts with rabbit muscle ATP-ase, which specifically cleaves the terminal high energy phosphate bond of ATP. For this, the perchloric acid extracts were neutralized to phenol red with KOH, the potassium perchlorate was allowed to precipitate for two hours in the cold and then centrifuged off. The clear supernatant fluid was made up to a volume with water, and then divided into two aliquots. The first aliquot was acidified with trichloroacetic acid and analyzed for total nucleotide labile P by the method described above, while the second aliquot was incubated for 10 min. at 25°C with rabbit muscle ATP-ase, previous addition of TRIS buffer pH 8.3 to a final concentration of 0.1 M, EDTA to a final concentration of 0.005 M and enough solid KCl to make a final concentration of 1 M. Rabbit muscle ATP-ase was prepared essentially according to Mommaerts and Parrish (11), up to step 4 of their method. This preparation contained some adenylate kinase activity, which was completely inhibited by EDTA in the conditions specified above, as stated by Bowen and Kerwin (3). The amount of ATP-ase added to the buffered extract was in large excess for a complete hydrolysis of the ATP present in the pea tissue extracts. The reaction was stopped by addition of TCA to a final concentration of 4 per cent, then the Norit A charcoal was added and analysis for high energy phosphate bonds was carried out in the way described; it was found

unnecessary to separate the small amount of myosin flocculated by the trichloroacetic acid.

The effects of auxin on high energy phosphate have been compared with the parallel effects on growth and oxygen uptake. These have been studied in experimental conditions as close as possible to those in which the action on ATP had been observed (temperature, age of the material, volume of medium per segment, auxin concentration). Growth has been determined as per cent increase in fresh weight. In the determinations of oxygen uptake auxin has been added from the side arm to the central vessel of the Warburg flasks, containing 10 segments in 5 ml. of distilled water, one hour after the respiratory activity of the segments had reached a practically constant rate.

When the effect of IAA, DNP and of IAA and DNP added simultaneously had to be tested, these reagents were tipped into the main compartment of the Warburg flasks from the side arm, after the oxygen uptake of all the flasks had reached a constant rate.

It is important to mention that in all of these experiments the basic medium in which the segments were floating during treatment has been plain distilled water. The use of buffered media was avoided, in order to eliminate a possible interference of energy utilization phenomena due to active salt uptake by the tissue.

Norit A was purchased from Bios. All other reagents used were Mercks's (Darmstadt) analytical grade products.

Abbreviations: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; \sim P, high energy-bond nucleotide phosphate; DNP, 2,4-dinitrophenol; IAA, indole-3-acetic acid; EDTA, ethylenediaminetetracetic acid, disodium salt (versene).

Results

A) *Auxin induced nucleotide labile P increase in isolated pea internode segments*

The object of a first series of experiments has been to collect a consistent number of data on the effects of auxin on the \sim P level, and, simultaneously, on respiration and growth. A period of treatment of 30 minutes was selected, as it allowed to observe a distinct effect on growth and oxygen consumption, while structural differences between the actively growing auxin treated segments and the controls could still be considered of minor importance. Moreover, it appeared from the results of a parallel investigation that the maximum effect of auxin on high energy phosphate metabolism were to be found in the period between 15 and 45 minutes from the beginning of the auxin treatment.

The results of several separate experiments, often run on different pea cultures, are shown in Table 1. The small differences in age, etc. between the different cultures account for the variability of the high energy phosphate contents in the controls: in fact, a satisfactory homogeneity is observed for

Table 1. *Effect of IAA on ~P content of etiolated pea internodes.*

Reference number of pea culture	Time of treatment minutes	~P micrograms per gram of initial wet weight		% increase over control
		Control	IAA	
1	15	26.7	30.4	13.9
2	15	20.8	22.4	7.8
3	15	20.6	21.6	4.9
				Mean: 8.8
1	30	25.6	31.8	24.5
4	30	20.7	23.0	11.5
5	30	27.3	33.5	23.0
6	30	29.4	36.2	22.9
7	30	29.3	34.0	15.5
8	30	25.8	28.2	9.2
9	30	23.1	26.7	15.5
10	30	25.0	26.9	7.6
11	30	26.2	27.9	6.5
12	30	22.2	24.8	11.7
13	30	18.2	20.4	12.0
14	30	18.4	19.8	7.5
15	30	19.1	20.5	7.3
				Mean: 13.4

Experimental conditions as described under "methods". Each value is the mean of duplicate experiments.

the data obtained from one culture. The effect of auxin in increasing the ~P level comes out quite neatly from these results, the average increase of ~P over the controls being of about 13 per cent. It has to be observed that in parallel experiments the changes induced by auxin on growth and respiration were determined, in experimental conditions substantially identical to those in which the effect on the ~P contents had appeared. In these experiments oxygen consumption was higher by about 19 per cent, and growth by about 98 per cent, in the auxin treated segments than in the controls: thus proving that the usual growth and respiration reactions to auxin were obtained under the experimental conditions in which the ~P response was detected.

The data of Table 1, although showing that IAA induces a significant increase of nucleotide acid labile phosphate, do not constitute in themselves an unequivocal demonstration that this is due to an increase of the ATP/ADP ratio; nor do they permit the calculation of the real entity of the ATP increase in the auxin treated segments. More information on this important point comes from the experiments of Table 2, in which nucleotide acid labile phosphate in the extracts has been determined not only as in the experiments of Table 1, but also after treatment of an aliquot of the extracts with rabbit muscle ATP-ase, which (in the presence of versene) specifically catalyses

Table 2. *The effect of IAA treatment on the ATP contents of etiolated pea internodes.*

Reference number of the pea culture	Treatment	~P $\mu\text{g}/\text{gram}$ of initial wet weight		ATP, $\mu\text{moles}/\text{gram}$ of initial w.w.
		Untreated extract	ATPase-treated extract	
16	Control	20.30	16.74	0.115
	IAA 5×10^{-5} M	22.90	16.74	0.198
17	Control	20.58	17.54	0.098
	IAA 5×10^{-5} M	24.58	17.60	0.226
18	Control	26.20	21.40	0.148
	IAA 5×10^{-5} M	30.80	21.40	0.303

Time of incubation of the segments in distilled water (controls) or in the IAA solution: 30 minutes. Each value represents the mean from three separate experiments. The ATP values of the third column have been calculated from the differences between the values of the first and the second column.

the hydrolytical cleavage of the terminal phosphate group of ATP. The difference between the two values thus obtained for each extract corresponds to the amount of ATP present (with the possible concurrence of small quantities of other ATP-ase cleaved nucleosidetriphosphates as guanosine- or inosinetriphosphate); a difference of $1 \mu\text{M}$ of labile P found corresponding to $1 \mu\text{M}$ of ATP in the perchloric extract.

It may be seen from Table 2 that the increase of the nucleotide acid labile P in the auxin treated segments is exclusively due to the terminal ATP phosphate: as identical values are found for the auxin treated segments and for the controls, when the ATP terminal P has been hydrolyzed, in the extracts, by the ATP-ase treatment. This indicates that the effect of auxin on nucleotide labile phosphate is indeed due to the capacity of the hormone to increase the ATP/ADP ratio, the total amount of nucleotides molecules remaining unchanged. Moreover, the relatively small difference in labile P between the auxin treated segments and the controls (+17 per cent) becomes much larger, on a percent basis, when expressed as a variation of ATP contents: the average auxin induced increase of ATP thus appearing of about 100 per cent.

B) *The effects of auxin on ATP contents as a function of time of treatment*

High energy phosphate determinations, as indicated above, were effected in segments maintained in distilled water, or, resp., in IAA solutions for periods of 30, 60, 120 and 240 minutes. As shown in Figure 1 high energy phosphate level (practically constant in the controls) in the presence of auxin significantly increased in the first period of the experiment, to decrease 90

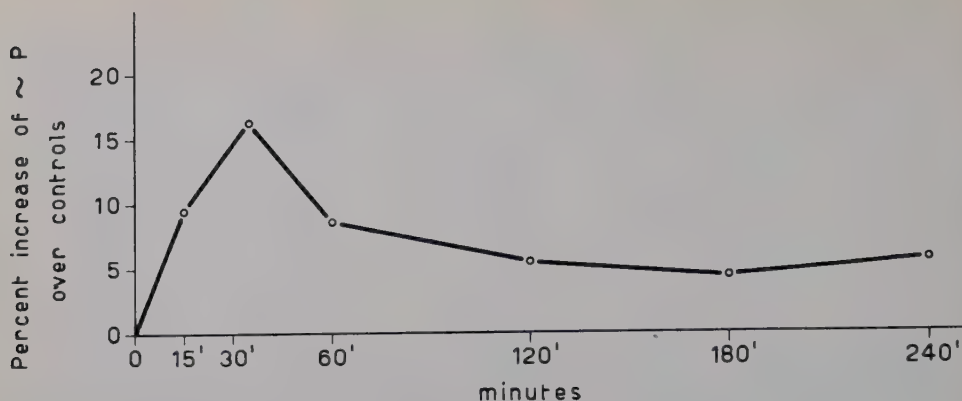


Figure 1. The effect of auxin ($\text{IAA } 10^{-4} \text{ M}$) on high energy phosphate content of pea internode segments as a function of time.

minutes after the beginning of the experiment to a value still slightly higher than that of the controls. This behaviour, which confirms the results described elsewhere (10), seems to indicate the existence, in the process of growth activation by auxin, of two different phases, characterized by a different equilibrium between the activities of the high energy bond producing and the high energy bond utilizing mechanisms (10). As far as the problem here investigated is concerned, the data of Figure 1 are of importance, inasmuch they show that the maximum effect of auxin on ATP must be searched in the very first period of the treatment, when the influence of secondary processes connected with cell growth and probably involving an increased ATP utilization are not yet of a determining importance.

C) The effects of auxin and DNP on respiration

The results of the experiments mentioned above showed that auxin at a concentration stimulating growth and respiration does also, at least in the first period of its action, induce a significant increase of the ATP/ADP ratio. This condition is incompatible with the view that the auxin induced respiratory rise is primarily due to the increased utilization, in the tissue stimulated to growth, of the high energy bonds of ATP: as it appears obvious that such a mechanism would be based, on the contrary, on a decrease of ATP and an increase of high energy phosphate acceptors.

On the other hand, the experimental basis of the hypothesis formulated by Bonner *et al.* (2) is mainly constituted by the finding that in the artichoke tuber tissue the large activating effect of IAA on respiration does not sum up

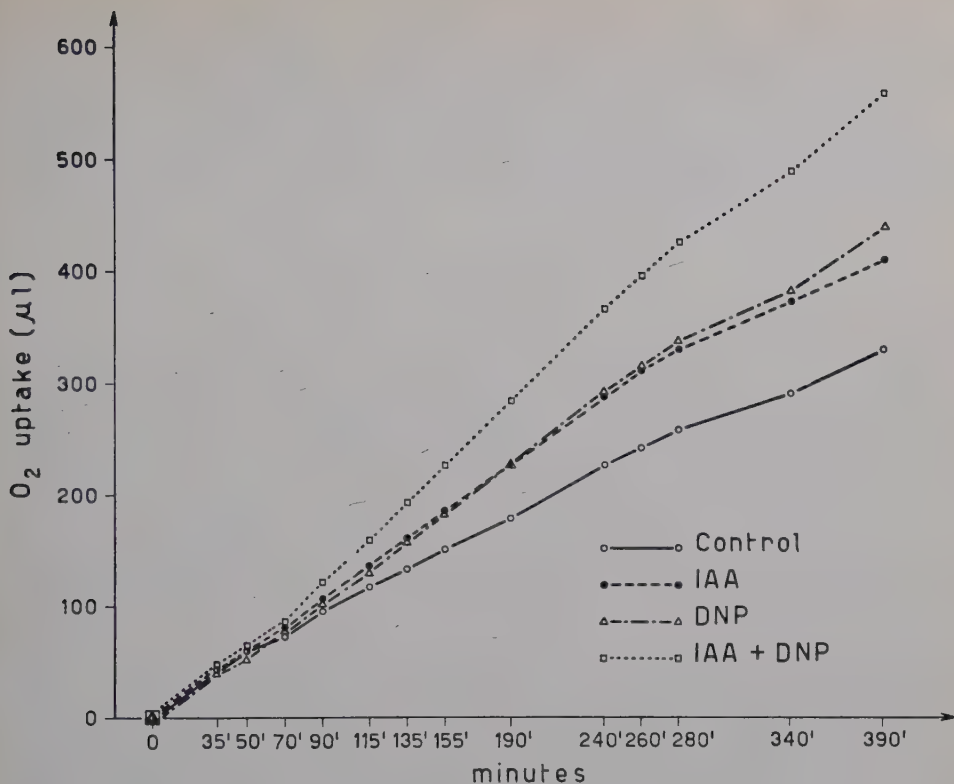


Figure 2. The effects of 10^{-4} M IAA, of 5×10^{-5} M DNP and of IAA and DNP together on oxygen uptake by excised pea internode segments.

with that of DNP, a reagent which is known to increase the oxidative metabolism, *in vivo* as in mitochondrial preparations, by uncoupling phosphorylations from oxidations, and thus by removing the limiting effect of phosphate acceptor concentration on the oxidative reactions.

It seemed therefore convenient to test the effects of DNP and of auxin, supplied separately and together, on the respiratory activity of our material, under experimental conditions comparable to those in which the effect of auxin on the ATP level had been detected.

The data of Figure 2 show that our results in this regard are completely different from those obtained by Bonner *et al.* on the artichoke tuber tissue. It may be seen, in fact, that the respiratory activation by DNP does in our case *almost exactly sum up with that observed in the presence of auxin*: and this for a period which lasts for no less than 6 hours from the beginning of the experiments. As both IAA and DNP have been used, in these experi-

ments, at their optimal respiration activating concentration, there appears to be little doubt left that at least in this case two different mechanisms are involved in the activation of O_2 uptake by the two compounds.

A possible explanation of the discrepancy between our results on the pea segments and those of Bonner *et al.* on the artichoke tuber tissue will be discussed in the following paragraph.

Discussion

The results of the experiments here reported may be summarized as follows:

1) Auxin at optimal growth and respiration stimulating concentration induces a definite increase of the ATP/ADP ratio in the excised pea internode tissue.

2) The $\sim P$ level in the auxin treated tissue is already significantly increased 15 minutes after the treatment is started, reaches its maximum around the 30th minute, then decreases again to a value slightly higher than that of the controls.

3) The stimulating effects of auxin and of DNP on the respiratory activity of pea internode segments (30 and 25 per cent stimulation respectively, for the two compounds tested separately) sum up almost exactly when the DNP and IAA are added simultaneously to the medium in which the segments are floating: thus indicating that different metabolic reactions are affected by these two reagents.

These results are interpreted as showing that the respiratory rise determined by auxin does not depend, at least in the first period of the cell reaction to the hormone, on the equilibrium state of the adenyl phosphate system: in other words, that it cannot be ascribed to an increase of phosphate ($\sim P$) acceptor level, consequent to the direct activation by auxin of some energy consuming process connected with growth, as suggested by Bonner *et al.* Consequently, the present data provide an indirect evidence in favour of a more direct activation by auxin of the enzymatic systems involved in oxidative metabolism, as dehydrogenases or other electron transferring enzymes, the activity of which would be a limiting factor for the respiratory rate of the tissues. The mechanism of this activation could be imagined, at the present state of knowledge, either to consist in a direct interaction between the hormone and these oxidative enzymes, or mediated through a primary auxin effect on such efficient regulating systems as glutathione or ascorbic acid; the latter hypothesis being, at the present time, supported by what seems to us a more convincing experimental basis (8).

On the other hand, this view is far from underestimating the importance of the equilibrium state of the adenyolphosphate system in the mechanism by which respiration and growth are controlled by auxin. On the contrary, it seems that at least an important point, in our data, does actually require, in order to be explained, the intervention of a mechanism in which a portion of the auxin induced respiratory rise is indeed determined by an increased ATP utilization in growth, and thus by the increased availability of $\sim P$ acceptor. In fact, a comparison between the behaviour of $\sim P$ and that of respiration as functions of the time of auxin treatment shows that at a moment, which in our experiments corresponds to 30 minutes after the treatment has started, $\sim P$ begin to decrease towards a value lower than the maximum, while O_2 consumption still steadily increases. It seems reasonable to assume that this $\sim P$ decrease is not imputable to a decreased efficiency of the phosphorylating process, but rather to an increased rate of an ATP utilizing reaction: that is to some kind of work connected with growth: as, possibly, osmotic work, or the reelaboration of plasma or cell wall structures. Now, as this decrease of ATP corresponds to an increased ADP level, the latter condition certainly appears such as to induce a supplementary rise of the oxidative rate, through a mechanism which, in this case, could be comparable to that of the respiratory activation by DNP. But the fact that this mechanism may, and probably does, play a rôle in the auxin effect of respiration, does not contradict the evidence here presented, that in its initial phase auxin activates oxidative metabolism not only independently from any increase of $\sim P$ acceptor; but, rather, against a decrease of the same, which is indicated by the increase of the ATP/ADP ratio.

On the other hand, an attentive consideration of the experiments on the artichoke tissue, on which Bonner's *et al.* (2) hypothesis is based, does not necessarily lead to conclusions inconciliable with our interpretation. It may be observed that in those experiments DNP has been supplied to tissues which had been treated with IAA in one case for 4, and in a second for 7 days. It seems hardly possible therefore, to sustain that in these conditions one is dealing with the primary effect of auxin on respiration. On the other hand it appears reasonable to assume that after a long period of auxin treatment metabolic work associated with growth (particularly in a tissue such as that of the artichoke tuber) should be very high, and thus the DNP sensitive, $\sim P$ acceptor limited fraction of respiration should be at its lowest level. Furthermore, it seems doubtful whether the absence, or the incompleteness, of additivity of the IAA and DNP effects on respiration necessarily means that the two reagents act on the same metabolic process: as one could think that some other limiting factor (as terminal oxidase activity, or substrate concentration, or O_2 availability) could impose to the respiratory rate a limit

well below that required by the theoretical sum of the DNP and IAA effects. In fact, from the experiments of Bonner *et al.* it may be observed that DNP and auxin even in the artichoke tissue, at some considerable extent do actually sum up, when IAA concentration is not so high as to provide its maximum (and really remarkable) stimulating effect on respiration.

Summary

The effects of auxin (IAA) at growth and respiration activating concentration, on high energy phosphate metabolism in excised pea internode segments have been studied. High energy phosphate ($\sim P$) has been determined as labile phosphate of nucleotides adsorbable on Norit A. Adenosine triphosphate has been determined by an enzymatic procedure utilizing rabbit muscle ATP-ase.

$\sim P$ level increased rapidly, in the auxin treated segments, during the first 30 minutes, to a level significantly higher than that in the controls. In the following period $\sim P$ decreased again to a value intermediate between the initial one and that of the controls.

The initial $\sim P$ increase appeared completely accounted for by the increase of ATP, the ATP contents in the segments treated with auxin for 30 minutes being about double than in the controls, while no change was observed for total Norit A adsorbable nucleotides; which indicates that auxin effects $\sim P$ metabolism by changing the ATP/ADP ratio.

In a second series of experiments the effects of IAA and of 2—4 DNP on the O_2 uptake by the excised pea internode segments was investigated. A marked activating effect of both reagents on respiration was observed. When IAA and 2,4-DNP were supplied together, the effect on O_2 uptake almost exactly corresponded, for a period of 6 hours, to the sum of the effects of the two reagents, when supplied separately.

These findings are interpreted as showing that the primary effect of auxin on oxidative metabolism does not depend on phosphate acceptor availability, but more probably involves the activation of oxidative enzymes. Only in a second phase of the auxin action a considerable part of the respiratory rise should be explained as a consequence of an increased utilization of high energy phosphate in metabolic processes involved in growth.

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Der Zusammenhang zwischen Fruchtkörperbildung und Atmung im Falle des Mutterkorns unter besonderer Berücksichtigung der Bedeutung der Fumarsäure

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Zur Anregung der Fruchtkörperbildung des Mutterkorns bedarf es einer Kältebehandlung von einigen Wochen. Viele haben sich bereits mit den genaueren Untersuchungen der Kältewirkung beschäftigt und konnten dabei die Feststellung machen, dass diese Behandlung eine Ähnlichkeit mit der Vernalisation von grünen Pflanzen aufweist; was jedoch der biochemische Hintergrund bei der Bildung von Fruchtkörpern des Mutterkorns sei, ist bisher von niemanden untersucht worden. Die Klärung dieser letztgenannten Frage soll nun der Zweck meiner Untersuchungen sein und das umso mehr, da gerade das Mutterkorn für solche Untersuchungen ein sehr günstiges Testmaterial darzustellen scheint. Da die Fruchtkörper ziemlich gross sind, lassen sich die vegetativen und generativen Teile leicht separieren, das Versuchsmaterial lässt sich gut aufbewahren und die Fruchtkörperbildung geht ziemlich rasch vonstatten. In diesem Aufsatz soll auf den zwischen den vegetativen und generativen Teilen des Mutterkorns auftretenden Unterschied der Atmung hingewiesen werden.

Als *Versuchsmaterial* diene ein von Petkuser Winterroggen geernteter Mutterkornstamm: Magyar 12. Bis November verblieben die Sklerotien in natürlichem trockenen Zustande im Samenkasten. Anfang Dezember wurden sie mit Lehm vermischt (für 1 kg Sklerotium wurde 6—8 kg Lehm gerechnet) und dies wurde dann in gewöhnliche Blumentöpfe gefüllt. 3—4 Tage verblieben die Blumentöpfe in feuchtem Zustande bei Zimmertemperatur; am 5. Dezember wurden sie in Freiland gesenkt. Am 15. Februar wurden die Blumentöpfe aufgenommen, der Lehm ausgewaschen und

Tab. 1. O_2 -Verbrauch (QO_2) und *Respirationsquotient* (RQ) von Sklerotien und Fruchtkörpern in verschiedenen Entwicklungszuständen.

Entwicklungszustand	Mit Kälte behandelt						Kontrolle			
	1	2	3		4		1	2	3	4
			ScL.	Fruchtk.	ScL.	Fruchtk.				
QO_2	23	29	60	347	76	338	41	39	17	19
RQ.....	0.68	0.65	0.67	0.94	0.67	0.93	0.69	0.67	0.68	0.67

die Sklerotien nach gründlichem Waschen unter Ausbreitung bei Zimmertemperatur getrocknet. Die so behandelten Sklerotien können im Samenkasten gelagert werden und bewahren ihre Keimfähigkeit 4—6 Monate lang in unverändertem Zustande.

Es ist jedoch zu bemerken, dass sich diese natürliche Art des Gefrierens von Sklerotien als bedeutend erfolgreicher erwiesen hat, als das Gefrieren im Eiskasten in einer Petrischale. Bei letzterem wurden die Sklerotien häufig von Pilzen und Bakterien angegriffen.

Die Keimung der Sklerotien fand in Petrischalen auf feuchtem Filterpapier statt. Mit dem Gefrieren wird — offenbar wegen Wetterfaktoren — nicht jedes Jahr der gleiche Erfolg erzielt. Abhängig davon setzt die Fruchtkörperbildung gewöhnlich 5—6, bzw. 10—15 Tage nach ihrer Unterbringung in der Petrischale ein und nach 25—50 Tagen erreichen die Fruchtkörper den Askosporen-Reifezustand. Früher liessen Boros und Garay (1955) die Sklerotien in Lehm, bzw. Moos keimen. Es hat sich aber herausgestellt, dass die Sklerotien in Petrischalen auf feuchtem Filterpapier ebenso gut keimen, wie in Lehm oder Moos. In Petrischalen mit einem Durchmesser von 10 cm wurden 8—10 Sklerotien gelegt. Die Keimung erfolgte bei Zimmertemperatur bei zerstreutem Licht.

Die den Fruchtkörper treibenden Sklerotien wurden in vier Entwicklungsstadien untersucht:

1. An den Sklerotien tritt der Ansatz der Fruchtkörper noch nicht in Erscheinung (2—5 Tage).
2. Der äussere Teil der Sklerotien springt auf und das Beginnen der Fruchtkörperbildung wird sichtbar (5—8 Tage).
3. Die jungen Fruchtkörper sind noch unreif (12—18 Tage).
4. Die Fruchtkörper sind im Stadium der Askosporenreife (23—30 Tage).

In untenangeführter Tabelle sind die einzelnen Entwicklungsstadien mit den Nummern 1—4 bezeichnet. Im Stadium 3 und 4 werden stets zwei Messungen durchgeführt. Zuerst wurde der gemeinsame Sauerstoffverbrauch von Sklerotien und Fruchtkörper und nachher der gesonderte Verbrauch nach der bei der Stielbasis erfolgten Abtrennung der Fruchtkörper vom Sklerotium, die Intensität der Atmung gemessen. Bei letzterer haben sich stets etwas höhere Werte ergeben, was wohl auf die Verwundungen, die bei der Abtrennung der Fruchtkörper entstehen, zurückzuführen ist. Bei einem Vergleich zahlreicher Messungen hat sich gezeigt, dass die Atmungsintensität durch erwähnte Verwundungen durchschnittlich um 11.6 prozent erhöht wird.

Ausserdem wurden in eine andere Petrischale auch nicht-gefrorene Sklerotien auf

feuchtes Filterpapier gelegt. Selbstverständlich trieben diese keine Fruchtkörper, ihre Atmung wurde jedoch in denselben Zeitabschnitten gemessen, wie dies bei den vier Stadien der gefrorenen Sklerotien geschah.

Methoden

Die Infiltrierung von Sklerotien und isolierten Fruchtkörpern wurde unter Vakuum bei 18—20 Hg mm solange fortgesetzt, bis aus den Sklerotien keine Luftblasen mehr hervorquollen und auf den Boden der Infiltrierflüssigkeit sanken. Dazu waren gewöhnlich 40—50 Minuten nötig. Nachher standen die Sklerotien vor Beginn der Messungen eine halbe Stunde lang auf Filterpapier.

Die Messung der Atmungsintensität wurde in einem Warburg-Apparat nach der üblichen Methode durchgeführt. Sämtliche in diesem Aufsatz angeführten Daten beziehen sich auf den Mittelwert von acht Messungen.

Resultate

Wie aus Abb. 1 hervorgeht, ist die Wassersaugung von gefrorenen Sklerotien, in dunstgesättigtem Luftraum gemessen, schneller und ihre Wasserkapazität höher als die der Kontroll-Sklerotien.

In Abb. 2 wurden der O_2 -Verbrauch und die CO_2 -Ausscheidung der Sklerotien, die die Fruchtkörper bilden und der Kontrolle dienen, dargestellt. Wie

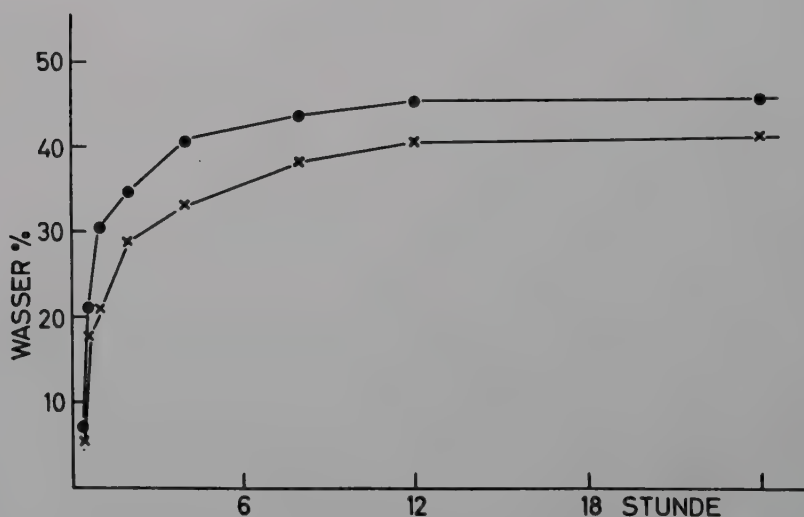


Abb. 1. Wassersaugung von gefrorenen (●—●—●) und Kontroll-Sklerotien (×—×—×) ausgedrückt im Prozentsatz des Wassergehaltes.

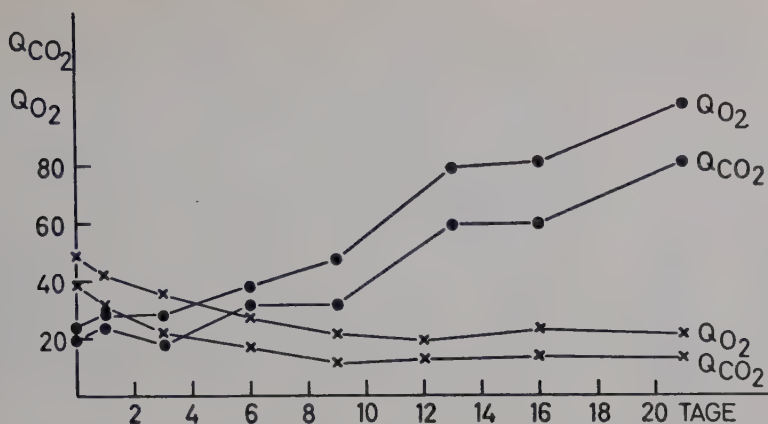


Abb. 2. Sauerstoffverbrauch von gefrorenen (●-●-●) und Kontroll-Sklerotien (×-×-×) und ihre CO_2 Erzeugung während der Fruchtkörperbildung.

zu erwarten war, geht aus den Resultaten hervor, dass sich der im Ruhezustand gemessene Wert der Atmungsintensität während der Fruchtkörperbildung auf das 4—5-fache erhöht. Auffallend ist dabei, dass die Atmung der Kontroll-Sklerotien bei Beginn der Untersuchung sehr intensiv ist, später aber wesentlich abnimmt. Eine Erklärung hierfür kann vorläufig nur auf Vermutungen beruhen, als wahrscheinlichste ist anzunehmen, dass die vom Roggen bereits trocken gesammelten Sklerotien eine gewisse Nachreife erfahren, sobald sie neuerdings in den angeschwollenen Zustand gelangen, und dies dann eine intensivere Atmung herbeiführt. Selbstverständlich muss diese Frage noch weiteren Untersuchungen unterworfen werden.

Angeichts dessen, dass die Daten des Grafikons die Gesamtatmung der Fruchtkörper angeben, erteilen sie keine Aufklärung bezüglich der Verteilung der während der Fruchtkörperbildung beobachteten Atmungserhöhung zwischen dem Sklerotium und den generativen Teilen. Darauf bezieht sich Tabelle 1.

Aus der Tabelle ist folgendes ersichtlich: Die Atmung der Fruchtkörper übertrifft den O_2 -Verbrauch der Sklerotien um vieles, ihr RQ ist jedoch 0.94. Demgegenüber erreicht der RQ der Sklerotien weder bei den gefrorenen, noch bei den für Kontrollen bestimmten 0.70 und obwohl sich während der Fruchtkörperbildung die Atmung der Sklerotien erhöht, bleibt ihr RQ unverändert. Die bereits hinsichtlich des RQ auftretenden bedeutenden Abweichungen weisen ebenfalls auf den zwischen der Atmung der Fruchtkörper und Sklerotien bestehenden bedeutenden Unterschied hin. Dies wird durch eine weitere Messung weit ausdrucksvoller nachgewiesen. In die sich in verschiedenen Entwicklungsstadien befindlichen Sklerotien wurden einzelne Säuren des Zitronen-

Tab. 2. *Wirkung der Infiltrierung von Säuren auf den O₂-Verbrauch (QO₂) der in verschiedenen Entwicklungsstadien befindlichen Sklerotien und Fruchtkörper.*

Infiltrierte Säuren $2 \cdot 10^{-2} M$	Entwicklungszustand									
	Mit kälte behandelt						Kontrolle			
	1	2	3		4		1	2	3	4
			Scl.	Fruchtk.	Scl.	Fruchtk.				
Dest. Wasser	24	28	49	381	58	329	34	29	19	20
Malonsäure	16	19	27	39	29	38	21	21	19	22
Fumarsäure	13	15	25	11	16	10	21	19	20	15
Bernsteins. ...	26	38	50	390	44	310	36	22	26	16
Apfelsäure ...	19	25	35	336	34	300	24	21	25	20
Zitronens.	19	20	47	314	35	296	21	22	20	19

säurenzykluses und als spezifischer Inhibitor Malonsäure infiltriert. In Tabelle 2 sind die Resultate angeführt.

Wie ersichtlich, hemmt die Malonsäure die Atmung der Sklerotien nur unbedeutend, hingegen vergiftet sie 89 prozent des O₂-Verbrauches der Fruchtkörper. Bernstein-, Apfel- und Zitronensäure üben keine wesentliche Wirkung aus, so ist es umso überraschender, dass sich die Fumarsäure, ähnlich wie die Malonsäure, als ein intensiverer Inhibitor erwiesen hat und eine 98 %ige Hemmung der Fruchtkörperatmung herbeiführte. Aus diesen Angaben geht deutlich hervor, dass die generativen Teile mit Bezug auf den Zitronensäure-Zyklus von den vegetativen Teilen Abweichen und ihr Sauerstoffwechsel von dem allgemein angenommenen Schema verschieden ist, da durch die Fumarsäure ihr O₂-Verbrauch vergiftet wird.

Zwecks Bekräftigung der auffallenden Wirkung der Fumarsäure wurden drei Präparate verschiedenen Ursprungs untersucht und bei sämtlichen konnte der obenerwähnte Effekt festgestellt werden. Ausserdem wurde die Reinheit der Fumarsäure auch mittels papierchromatographischer Untersuchungen kontrolliert. Gleichzeitig wurde die auf die Saprophytenkultur des Mutterkorns ausgeübte Wirkung der Fumarsäure geprüft. Ich konnte feststellen, dass in Gegenwart von Fumarsäure das Wachstum der Saprophytenkultur mit der Kontrolle vollkommen übereinstimmte. Die von Garay (1956) bekanntgegebene Grundnährlösung fand bei diesen Untersuchungen Verwendung.

Die Fumarsäure scheint also den Stoffwechsel vegetativer Teile nicht zu beeinflussen, während sie jedoch auf den Stoffwechsel der generativen Teile als starker Inhibitor wirkt. Um in diese Frage einen tieferen Einblick zu erlangen, wurde folgender Versuch angestellt: Bevor die Sklerotien zu keimen begannen, wurden ihnen die entsprechenden Säuren bzw. deren Kombination infiltriert und ihre Fruchtkörperbildung und Atmung studiert. Kolonne A der

Tab. 3. Wirkung der Infiltrierung verschiedener Säuren auf die Fruchtkörperbildung und Atmung der Sklerotien (A), sowie auf die Atmung der Fruchtkörper (B).

Infiltrierte Säure $2 \cdot 10^{-2} M$	A. Die Sklerotien (18 Tage nach der Infiltrierung)			B. Frucht- körper
	Fruchtkörper Trieben %	Zahl der auf ein Sklerotium fallenden Köpfe	QO ₂	QO ₂
Dest. Wasser	63	10	114 ¹	342
Malonsäure (M)	0	0	29	38
Fumarsäure (F)	0	0	20	20
Bernsteinsäure (B)	60	8	101 ¹	324
Apfelsäure (A)	17	3	36 ¹	312
Zitronensäure (Z)	11	2	47 ¹	327
M+B	25	6	54 ¹	267
M+A	0	0	33	39
M+Z	0	0	28	27
F+B	9	3	39 ¹	245
F+A	0	0	26	19
F+Z	0	0	26	17

¹ Sclerotien mit den Fruchtkörpern.

Tab. 3 gibt die Resultate an. Wie ersichtlich, wird die Entstehung der Fruchtkörper durch die Malon- und Fumarsäure 100 %ig gehemmt und auch im Falle der Apfel- sowie Zitronensäure sind bedeutende Hemmungen zu beobachten, während die Bernsteinsäure die Fruchtkörperbildung nicht beeinflusst. Gleichzeitig geht aus der Tabelle hervor, dass die Giftwirkung der Malon- und Fumarsäure von der Bernsteinsäure teilweise kompensiert wird und zwar konnte mittels der Bernsteinsäure die Inhibition der Malonsäure in grösserem Masse (42 %) aufgehoben werden als die der Fumarsäure (15 %), was mit den bereits erwähnten Daten in guter Übereinstimmung steht, wonach die Fumarsäure eine grössere inhibierende Wirkung auf die Atmung der Fruchtkörper ausübt als die Malonsäure.

In Rubrik B der Tab. 3 wurde die auf die Atmung der Fruchtkörper ausgeübte Wirkung verschiedener Infiltrierungen angegeben. Wie aus obigen Angaben zu erwarten war, wurde die inhibierende Wirkung der Malon- und Fumarsäure von der Bernsteinsäure teilweise (72 bis 78 %ig) aufgehoben. Mittels Zitronen- und Apfelsäure vermochte diese inhibierende Wirkung nicht kompensiert zu werden. Ein Widerspruch scheint sich bei einem Vergleich von Rubrik A und B der Tab. 3 zu ergeben, da hier die Apfel- und Zitronensäure die Fruchtkörperbildung wesentlich hemmen, während dieselben Säuren auf die Atmung der Fruchtkörper keine Wirkung ausüben. Obwohl es noch verfrüht wäre, diesbezüglich ein endgültiges Urteil zu fällen, ist anzunehmen, dass sich im Sklerotium aus der Zitronen- und Apfelsäure innerhalb einiger Tage Fumarsäure bildet und so die beobachtete Inhibition nur eine

indirekte sei, während bei einer Messung der Atmung von kurzer Zeitdauer eine derartige indirekte Wirkung nicht beobachtet wird. Diese Annahme darf selbstverständlich als blosser Arbeitshypothese aufgefasst werden.

Endlich muss es noch bemerkt werden, dass die kompensierende Wirkung der Bernsteinsäure ziemlich grosse Schwankungen aufwies. Manchmal war die Hemmungsaufhebung 71—78 prozent, manchmal nur 30—35 prozent.

Diskussion

Vorläufig wäre es noch verfrüht, aus den angeführten Daten weitgehende Schlüsse zu ziehen, mit Sicherheit kann man aber feststellen, dass zwischen der Atmung der vegetativen und generativen Teile des Mutterkorns wesentliche qualitative Unterschiede bestehen und die Fumarsäure, die die Atmung der Fruchtkörper vergiftet, gleichzeitig auch die Fruchtkörperbildung der Sklerotien hemmt. Nach Liverman und Bonner (1953) wird das Blühen der *Xanthium* sp. unter gewissen Bedingungen von den Säuren des Krebschen Zyklus vorteilhaft beeinflusst. Cantino und Hyatt (1953) haben nachgewiesen, dass bei dem einen Mutanten der *Blastocladiella* sp. gewisse Differenzierungsprozesse bei einem Ausfall der α -Ketoglutarase und Aconitase ausbleiben. Durch die Daten dieses Aufsatzes wird der bestehende enge Zusammenhang zwischen Säurezyklus und Morphogenese besonders betont.

Zusammenfassung

1. Die Wasserkapazität gefrorener Sklerotien ist grösser und ihre Wasser-saugung ist schneller als die der unbehandelten.
2. Während der Fruchtkörperbildung steigt die Atmungsintensität der Sklerotien um den zwei-dreifachen, im Ruhestand gemessenen Wert an.
3. Der RQ der Fruchtkörper ist 0.94, ihre Atmung ist bedeutend intensiver als die der Sklerotien. Die RQ der Sklerotien ist 0.70.
4. Die Malonsäure, ganz besonders aber die Fumarsäure hemmt den O_2 -Verbrauch der Fruchtkörper, während sie den der Sklerotien nicht wesentlich beeinflussen.
5. Mittels der Malon- und Fumarsäure ist es möglich, die Fruchtkörperbildung des Mutterkorns vollkommen zu hemmen.
6. Die in vorhergehenden Punkten erwähnte inhibierende Wirkung kann durch die Bernsteinsäure aufgehoben werden.
7. Zitronen- und Apfelsäure beeinflussen die Atmung der Fruchtkörper nicht, üben aber auf die Fruchtkörperbildung eine schwache inhibierende Wirkung aus.

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Transpiration as a Requirement for Growth of Land Plants

By

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Introduction

It long has been known that land plants lose relatively large quantities of water by evaporation from aerial portions, especially leaves (Hales, 2). This process, transpiration, appears to be detrimental to plants in that unless water is constantly supplied to the roots, death by desiccation certainly will follow. Because of this, the geographical distribution of plant species may be largely limited by amount of rainfall. Also plants are subject to damage or death by drought or other agencies that limit water supply.

Transpiration appears to be unavoidable. Plant functions require exchange of gaseous carbon dioxide and oxygen. This is largely limited to stomata and the largest portion of the surface of most land plants is of such a nature so as to reduce water loss to a minimum and transpiration occurs simply because they have evolved no method of exchanging certain gasses while excluding others, namely water vapor. For these and other reasons, many modern botany textbooks describe transpiration as being a necessary evil.

The movement of water through plants bodies is known to occur in both land plants, (Hales, 2) and aquatics, (Thut, 4). In land plants this movement is referred to as the transpiration stream and is felt to be a result of both transpiration and root pressure. The movement in submerged aquatics seems to be due to root pressure alone. It is generally conceded that inorganic salts are translocated in the transpiration stream and mineral uptake has been cor-

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related with transpiration (Hylmö), 3). However, so far as the writer is aware, no evidence has been presented to show that transpiration is necessary for growth or higher plants.

It is the purpose of the present study to present evidence that transpiration must occur for growth of some higher land plants by demonstrating that in the absence of transpiration growth does not occur or that under conditions of greatly reduced transpiration, growth rates are considerably reduced.

Methods

Plant growth as affected by high relative humidity and light was observed in each of four environmental conditions. The first two conditions were rooms where temperature was kept at 26°C within a degree and all light was excluded except a dim red one which was used only when necessary to observe experimental plants. Relative humidity was maintained by steam and in the first room was kept at about 87 %. This will be referred to as the dark room. In the second room relative humidity was maintained as close to 100 per cent as possible. This room will be referred to as the humid dark room. Plants placed in the humid dark room became a little damp, but never what one would refer to as wet.

The third environmental condition was a greenhouse where temperature, relative humidity and daylight were not controlled or measured. However the environment was generally favorable for plant growth.

The fourth condition was a separate chamber of the greenhouse enclosed with glass. Here temperature and daylight likewise were not controlled or measured, but the relative humidity was kept as high as possible by sprays that delivered a fine mist of tap water. Plants placed under this condition became quite wet on the upper surfaces. This environment will be referred to as the humid chamber of the greenhouse.

Including light as a factor, was an attempt to reduce temperature effects due to light absorption and also to exaggerate plant growth differences by etiolation.

Experimental

Experiment 1. The effect of extreme high humidity and light on growth of one-year-old Hardy pear trees.

Scions of Hardy pear were grafted on quince root stocks and grown during the spring and summer of 1954. At the end of the growth season the trees were about a meter high, unbranched and bearing only vegetative buds. Then they were placed with roots in soil bins in a heated greenhouse where they were kept dormant. On 11/9/55, the stems of four groups of five trees each, were immersed in tap water at 50°C for a half of an hour. This is known to force bud growth. Then the stems of two of each group of five trees were cut off about 15 cm. above the graft union. After this treatment, one group was placed in each of the four environmental condi-

tions described with the roots of each tree in aerated culture solution. After twenty-seven days, the group in the humid dark room was transferred to the dark room and the group in the humid chamber of the greenhouse was transferred to the normal greenhouse environment.

The trees placed in the greenhouse and in the dark room opened most of their buds within a week or two after the hot water treatment. At the end of 27 days, the leaves of the trees in the greenhouse were green and well expanded and a comparatively slight amount of shoot extension was observed. The trees in the dark room had comparatively smaller white or yellowish leaves and much etiolated shoot growth.

The trees placed in the humid chamber of the greenhouse and in the humid dark room showed no bud growth at the end of 27 days. Upon being transferred to the dark room from the humid dark room and to the greenhouse from the humid chamber of the greenhouse, both groups of trees showed bud growth within two weeks.

Experiment 2. The effect of extreme high humidity and light on germinating sunflower seeds.

On 11/8/55, sunflower seeds of the Russian Mammoth variety were planted in pots of sand and placed in each of the four experimental conditions described. They were watered only as often as necessary to keep the sand moist. Fourteen days later, observations of the seeds placed in the dark room and the humid dark room were made and seedling growth was measured as the length from the base of the cotyledons to the tip of the shoot. Although considerable growth of hypocotyls occurred, this was omitted from measurements for convenience. Twenty-seven days later similar measurements were made of the growth of seeds placed in the greenhouse and in the humid chamber of the greenhouse.

During the fourteen day period, seeds placed in the dark room and the humid dark room germinated and grew. Measurements of shoot growth showed an average of 6 cm. for 109 seedlings grown in the dark room and 3 cm. for 80 seedlings grown in the humid dark room. The seedlings grown in the dark room had light yellow cotyledons. Those grown in the humid dark room had deep yellow cotyledons and lateral rootlets appearing on the hypocotyl well above the level of the sand in which they were planted.

During the twenty-seven day period, seeds placed in the greenhouse and the humid chamber of the greenhouse likewise germinated and grew. Measurements showed an average of 6 cm. shoot growth by 129 seedlings grown in the greenhouse and 2 cm. by 129 seedlings grown in the humid chamber of the greenhouse. The cotyledons of seedlings grown in the greenhouse were light green. The seedlings grown in the humid chamber of the greenhouse had dark green cotyledons and lateral rootlets appearing on the hypocotyls.

Compared to seedlings germinated in the dark room and the greenhouse, the seedlings germinated in the humid dark room and the humid chamber of the greenhouse were observed to be shorter in new shoot growth but about equal in hypocotyl growth. Hypocotyl growth in all cases was roughly 15 to 25 cm.

Experiment 3. The effect of extreme high relative humidity and light on the growth of sunflower plants.

On 11/8/55, Russian Mammoth sunflower seeds were planted in pots of moist sand. Upon germination they were set out in a greenhouse with roots in aerated water culture. When the plants were one month old, they were placed in each of the four experimental conditions. At this time they were about 25 cm. high which is only a portion of the height of a mature plant. Nineteen days later, growth made by the plants was measured as the length from the base of the cotyledons to the tip of the shoot.

The plants in the dark room and the humid dark room died within six days, probably from starvation.

During the nineteen day period, plants in the greenhouse added from 21 to 49 cm. new shoot growth with a mean of 40 cm., while plants in the humid chamber of the greenhouse added from 9 to 26 cm. new shoot growth with a mean of 18 cm. On the average, only eleven plants were sampled in each locality which is a rather low number to allow much significance to the values of the means. However, in light of the effects of humidity on growth shown in experiments 1 and 2, it seems probable that the means are significantly different. The plants in the humid chamber of the greenhouse developed lateral rootlets on the hypocotyls. Aside from this and the lesser growth rate, the plants appeared quite normal. Their leaves remained as green as those on plants in the greenhouse and no apparent signs of mineral deficiencies were noted.

Summary of Results

Under environmental conditions of extreme high humidity, the growth of Hardy pear buds was completely checked, the growth rate of germinating sunflower seedlings was reduced to about half its normal value and the growth rate of young sunflower plants likewise was reduced to about half its normal value. This reduction of growth was not accompanied by leaf chlorosis or mineral deficiency symptoms.

Discussion

Although transpiration was probably reduced to relatively low levels by the high humidities, it is doubtful that it was completely stopped. Main-

tenance of 100 per cent relative humidity was not accomplished and further, considerable flow of water could occur at high extreme relative humidity if temperature gradients existed. For this reason it is assumed that transpiration did occur to some extent under experimental conditions of high extreme relative humidity and that were transpiration completely stopped, growth also would have completely stopped or almost so.

No studies were made to disclose the mechanism by which transpiration facilitates growth. However water movement and translocation were probably involved. Reduced translocation rates of organic and inorganic solutes might be expected to slow growth but only that translocation between adjacent cells and with cells. Mass translocation, such as from root to shoot, although known to be related to transpiration, would not seem to be a logical complete explanation for slowed growth rates at extreme high relative humidity. Pear buds are known to be well supplied with food and nutrients (Hahne, 1) and therefore should not be dependent upon the transpiration stream for such, at least for bud opening. Further, if poor supply of food or nutrients was the cause for the reduced growth rate of the sunflower plants, one would have expected signs of deficiencies. But the sunflower leaves remained green and the plants appeared normal in other respects throughout the course of the experiment, the length of which was adequate to show symptoms of deficiencies of a magnitude adequate to reduce the growth rate one half.

As mentioned, transpiration, while known to be related to water and salt movement within plants, has not been felt to be a function required for growth. The present experiments indicate that while transpiration normally might be excessive, it is a necessary function and must occur for the growth of some higher land plants.

The speculation that transpiration is responsible for translocation of organic and inorganic materials between adjacent cells and within cells is important because it would assign transpiration as the energy source for all translocation save that which might occur by diffusion.

Summary

Under experimental conditions of extreme high relative humidity, the growth of Hardy pear buds was completely checked and the growth rates of germinating sunflower seedlings and of young sunflower plants were reduced to about half their normal value. This reduction of growth was not accompanied by leaf chlorosis or mineral deficiency symptoms. It was concluded that transpiration is a necessary function of some higher land plants and must occur

for growth. It was suggested that transpiration is the energy source for both organic and inorganic solute translocation.

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The Antagonism of Auxin and Kinetin in Apical Dominance

By

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Although it is more than 20 years since it was shown that apical dominance is mediated by auxin, the method by which this is brought about remains obscure. At one time no less than nine theories were prevalent (see Thimann 1939) and at least one other has since been added (Libbert 1954 a). Although some of these have now been excluded, no one has been definitely supported, and it has seemed for some time that a fresh approach to the problem is called for.

Such a fresh approach has now become possible through a discovery made with plant tissue cultures. Skoog and Tsui (1948, 1951) have shown that the formation of buds in cultures of the pith of *Nicotiana tabacum* is controlled not only by auxin but also by the purine content, especially adenine, in the medium. In particular, kinetin, 6-furfurylaminopurine (Miller *et al.* 1955) allows the production on one tissue culture fragment of very large numbers of buds (Skoog and Miller 1957). Since these elongate side by side it follows that they are not strongly inhibiting one another's development. It seemed that the action of kinetin might therefore be exerted not only on the initiation of the buds but on their development as a whole. It was therefore thought of importance to study the effect of kinetin, not on bud formation, but on the growth and development of previously formed buds.

The present paper describes experiments on the lateral buds of *Pisum sativum* and brings for the first time evidence that the inhibiting action of auxin can be completely and quantitatively antagonized. A preliminary report of this work has been given (Wickson and Thimann 1956).

Materials and Methods

Pisum sativum var. Alaska was used as the test-plant. Seeds were germinated at 25°C and grown in water in a room maintained at 85 per cent relative humidity with occasional red light. They were thus not quite completely etiolated.

When the seedlings were 7 days old, sections 3.5 cm long were cut from the stem from just above the first (lowest) node to just above the second. The resulting sections, comprising one node and one internode, were immediately laid in sterile petri dishes each containing 10 ml of solution. In this way the sections were half-immersed but care was taken to keep the bud itself out of the solution. After a number of trials it was found that the control buds grew most satisfactorily in 1 per cent sucrose in bright light. The experimental solutions, therefore, usually contained 1 per cent sucrose and the dishes were kept in a light room (at 19°C) at high light intensity. Since two buds, one large and one small, are present at the second node, it was found necessary to pull off the scale leaf at the node after 2 to 3 days, and excise the smaller of the two buds. If this precaution were omitted, the development of the larger bud was irregular. To determine the extent of development of the bud, the stem-section was laid on a sheet of glass over graph-paper, and the length of the bud measured from its extreme tip to the point of insertion of the scale-leaf. Such measurements were usually made every 2 days if it were required to determine the growth rate. Otherwise a single reading was made after 5 to 8 days. Each result represents the average length of 10 buds.

Results

A. *The inhibiting action of auxin*

Figure 1 presents the course of growth of the lateral buds in a typical experiment. At the end of 5 days the control buds were growing rapidly while those in indoleacetic acid (henceforth referred to as IAA) at 1 mg or 10 mg per l. were essentially the same length as at the start. In low IAA concentration, however, the buds may begin to elongate after 5 days, a phenomenon which will be discussed below.

Naphthalene-1-acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4 D) had the same inhibiting action as IAA. Figure 2 illustrates their effects in an experiment similar to that of figure 1.

The inhibition is not due to poisoning, since buds in IAA grow normally after transference to plain sucrose. For example, a bud that after 5 days in IAA (4 mg per l.) still had its initial length of 0.8 mm, grew on being transferred to plain sucrose to a length of 5.7 mm in 5 days. Controls grew 4.1 mm in the same time. Furthermore the inhibition in these experiments resembles that occurring in intact plants in that it is dependent on continuous presence of the auxin. If IAA is applied 24 hours after separating the stem sections from the parent plant, the buds show a very slight retardation but thereafter

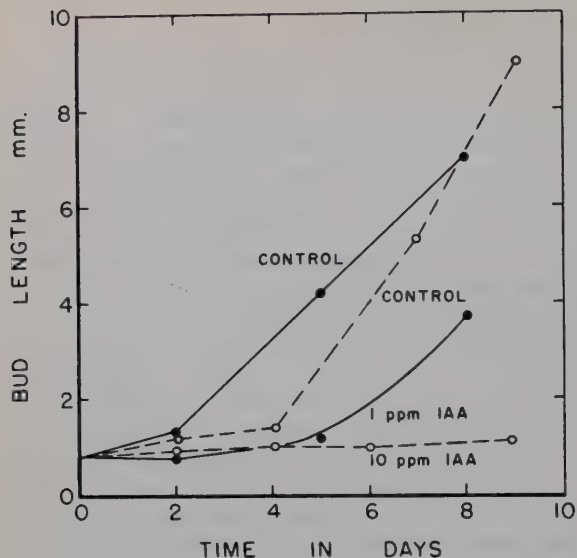


Figure 1. Effect of indoleacetic acid on growth of lateral buds. Basal medium 1 % sucrose. ○—○ growth in presence or absence of 10 ppm. IAA. ●—● Similar experiment performed with 1 ppm IAA.

they grow at the same rate as controls or even faster (Figure 3). Thimann and Skoog (1934) showed similarly with *Vicia Faba* that if the auxin supply was interrupted for as little as 12 hours the lateral buds would begin growth. Libbert (1954b) has made use of the same phenomenon by decapitating whole plants and at once grafting the terminal bud on again; the temporary interruption of the auxin flow due to the time required for the graft to take allows the lateral buds to escape from inhibition. This phenomenon shows

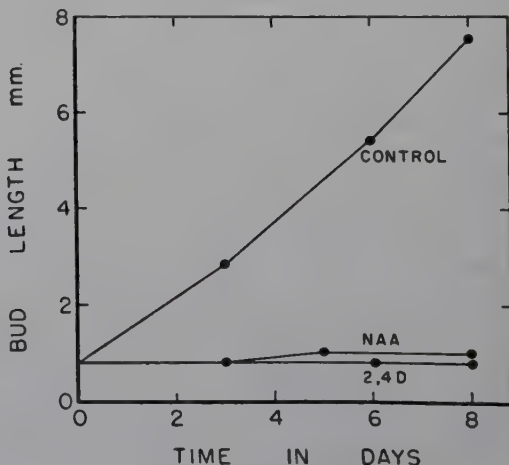
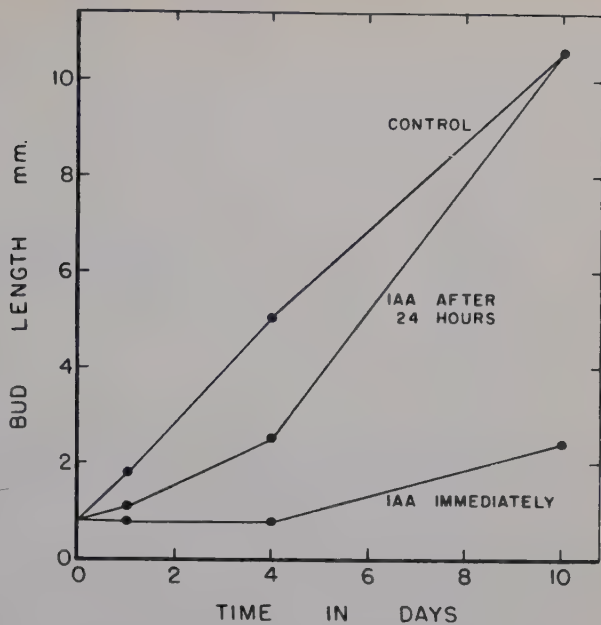


Figure 2. Effect of synthetic auxins on growth of lateral buds. Basal medium 1 % sucrose. Experimental solutions: 1 ppm naphthalene acetic acid or 1 ppm 2,4 dichlorophenoxyacetic acid.

Figure 3. *Effect of time-lapse between excision of stem-section from parent plant and its treatment with indoleacetic acid. Basal medium 1 % sucrose. Sections put into 4 ppm IAA either immediately on cutting or after 24 hours.*



that the release of buds from inhibition is a fundamental and sudden change, — the overcoming of some block which cannot be reinstated — rather than the general promotion of their elongation.

B. *The interaction between Auxin and Kinetin*

When kinetin is added to the medium together with auxin the inhibition is no longer exerted. Figure 4 illustrates such an experiment. Although the inhibition by indoleacetic acid alone is virtually complete, yet in the presence of IAA and kinetin the buds grow at the same speed as the controls. It will be noted that no delay is involved (as for instance in Figure 6 3), but the buds grow at the full rate from the start.

Kinetin alone has no very marked growth-promoting effect under the same conditions, *i.e.* in 1 per cent sucrose in light. The influence of a series of concentrations is summarized in Figure 5, in which the bud lengths after 8 days are plotted. An optimum increase of about 25 per cent in length is given by a concentration of 4 mg kinetin per liter ($\approx 2.10^{-5} M$). However, in the absence of sucrose the control buds grow very much less, and here the addition of kinetin does bring about considerable elongation (Figure 5) though it does not quite raise the growth to the level of that in sucrose.

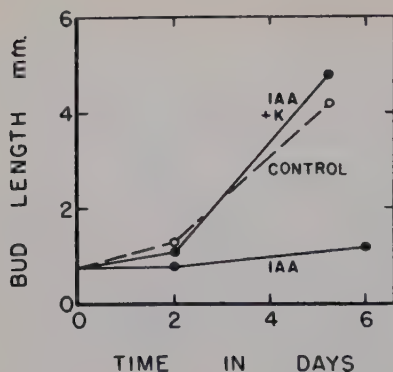


Figure 4. Interaction between indoleacetic acid and kinetin. Basal medium 1 % sucrose. Experimental solutions: 1 ppm IAA; 4 ppm kinetin.

Much more striking, however, is the effect of kinetin in darkness, where the control buds scarcely grow at all. At the optimum kinetin concentration of 5 ppm ($2.3 \cdot 10^{-5} M$) the buds elongate as rapidly as in the light.

Many experiments have been carried out to determine the ratio of concentrations of auxin and kinetin necessary for complete antagonism. Over the range from 0.3 to 5 mg. IAA per liter, concentrations of kinetin from 0.5 to 10 mg per liter added simultaneously gave more or less complete release of inhibition with an optimum concentration of the same order of magnitude as that of the auxin. Since the molecular weights are also of the same order

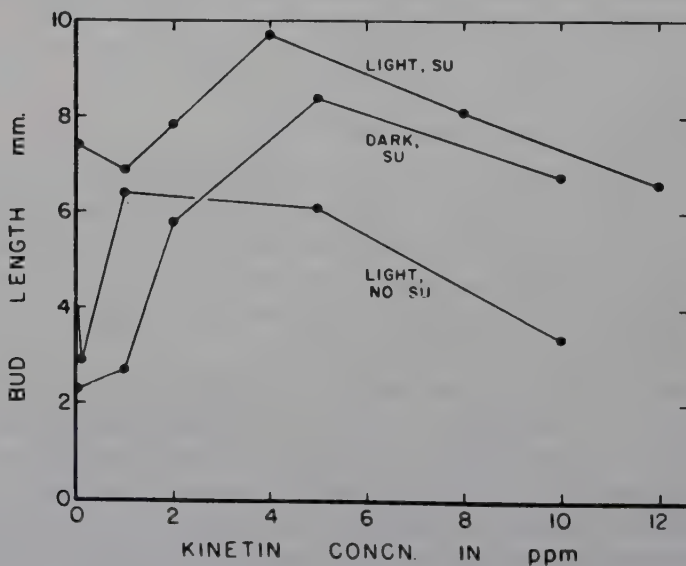


Figure 5. Effect of kinetin alone on growth of lateral buds. Experimental conditions: light=continuous light; dark=continuous dark; SU=1 % sucrose.

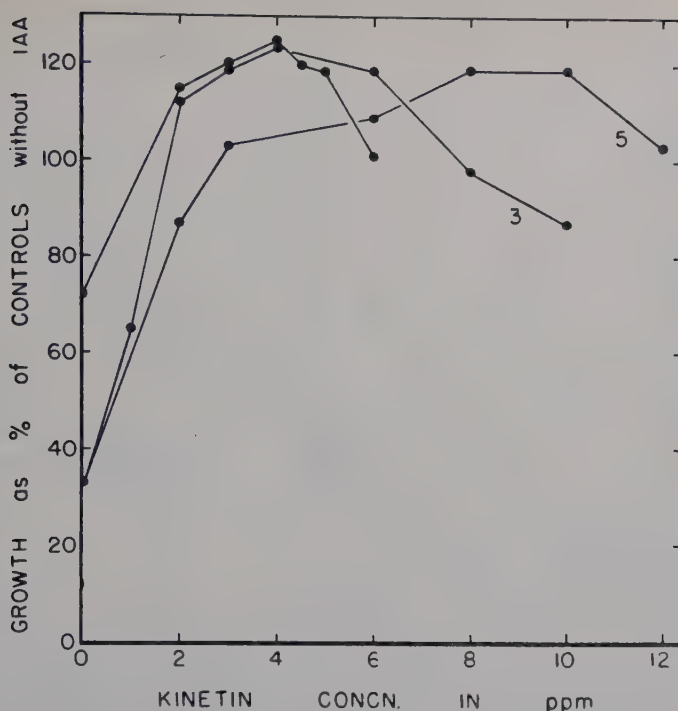


Figure 6. Growth of lateral buds in different mixtures of indoleacetic acid and kinetin. Basal medium: 1 % sucrose. Experiments carried out in 1, 3, or 5 ppm IAA.

(IAA=175, kinetin=217) this suggests roughly a 1 : 1 molar ratio. Figure 6 presents a series of experiments at different IAA concentrations, showing that as the auxin concentration increases, the concentration of kinetin necessary to obtain optimum growth also increases. At 15 mg IAA per liter 9 ppm of kinetin sufficed for reversal but a clear optimum was not reached.

It is difficult to assign a precise optimum kinetin concentration to each concentration of auxin, partly because the curves are somewhat flat-topped and the exact position of the optimum is obscured by minor fluctuations. It appears also that, in different experiments, the greater the absolute elongation of the buds in auxin alone, the lower was the concentration of kinetin giving optimum growth. This certainly suggests that variations in the normal growth of the buds may be due to natural variations in their content of a kinetin-like substance.

C. The "Breakaway"

It will have been noticed in Figure 1 that the growth of buds in 1 ppm IAA was almost totally arrested for the first 5 days of the experiment, but there-

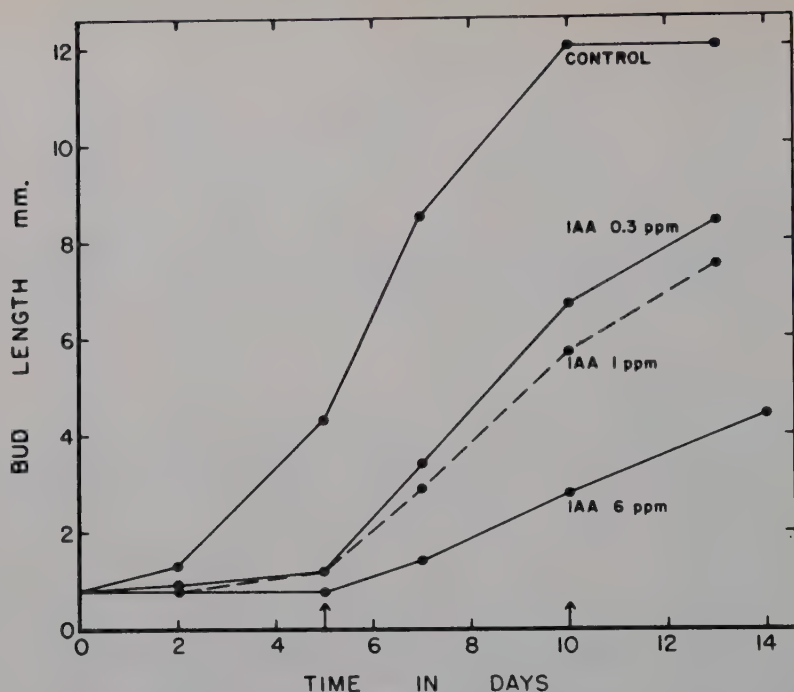


Figure 7. Growth of lateral buds in presence of indoleacetic acid. Basal medium: 1 % sucrose. Solutions renewed at points marked by arrows.

after they grew at almost the same rate as the controls. This sudden inception of growth was found to be of invariable occurrence at all concentrations of IAA up to about 8 ppm. The rate of growth after this 'breakaway' depended upon the concentration of IAA used; at low concentrations the rates were practically the same in the experimental and control buds, but above 1 mg per liter IAA there was some retardation which increased with increasing concentration of IAA (Figure 7). Went (1939) has also observed that the buds on isolated pea stem sections do commence growth after some days in presence of indoleacetic acid.

The "breakaway" is not due to an exhaustion of the supply of IAA since it was not delayed or even interrupted when the solutions were renewed (Figure 7). It could also be demonstrated in solutions of NAA and 2,4 D, but only provided their concentrations were not higher than 0.1 ppm (Figure 8); at higher concentrations these compounds inhibited growth totally. Such a difference in sensitivity between IAA and synthetic auxins certainly suggests a connection with the specific IAA-destroying enzyme, though it does not prove it.

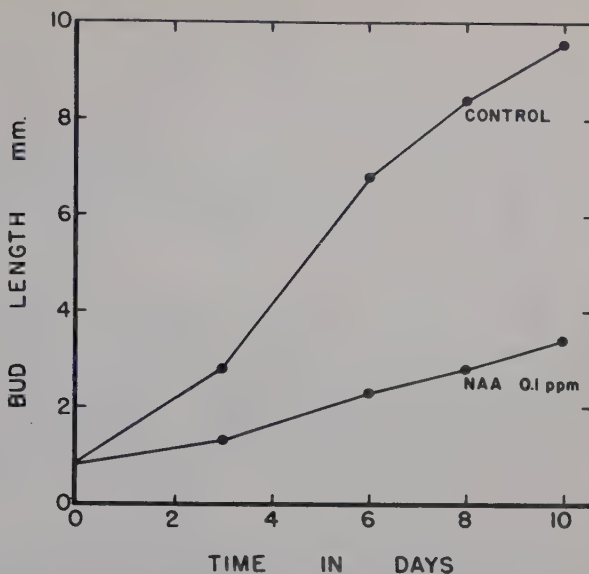


Figure 8. Growth of lateral buds in presence of naphthalene acetic acid. Basal medium 1 % sucrose.

The daylength in which the experiments take place appears to affect strongly the amount of "breakaway" which occurs. Experiments in 8-hour days, but otherwise similar to the usual ones in continuous light, showed little or no bud growth in IAA (3 mg per liter) even after 8 days. The curves for such short-day experiments resemble those shown for NAA and 2,4-D in Figure 2. That this difference is a photoperiodic phenomenon is shown by the fact that interruption of the long night by 20 minutes light reinstates the breakaway. For example after 8 days in 3 mg per liter IAA the length of buds as a percentage of untreated controls was: continuous light —25 per cent; short days —9 per cent; short days with interrupted nights —55 per cent.

D. Effect of gibberellic acid on bud development

The report of Brian *et al.* (1955) that pea seedlings treated with gibberellic acid ($G.A_3$) showed "noticeable increase in the length of lateral shoots", and the earlier observation of Kato (1953) on its stimulation of the buds in the axils of the cotyledons, led us to test $G.A_3$ for a possible role in apical dominance. At concentrations from 0.1 to 10 ppm $G.A_3$ was found to promote bud elongation under all the conditions tried. Figure 9 shows the effects of $G.A_3$ or kinetin tested in presence of sucrose. It will be seen that 5 ppm $G.A_3$ causes a growth promotion of 217 per cent; the comparable figure for kinetin in this experiment is only 6 per cent. It is clear that $G.A_3$ has a very great

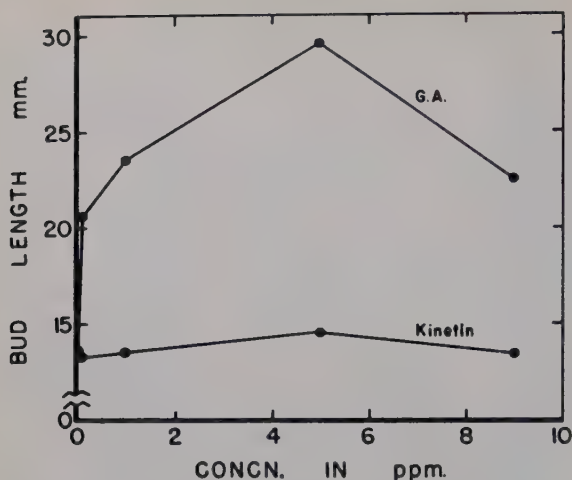


Figure 9. Comparison of effects of different concentrations of kinetin and gibberellic acid on growth of lateral buds. Basal medium 1 % sucrose.

innate ability to promote the elongation of buds, just as it has on intact stems. The elongation of 30 mm shown in figure 9 is the maximum obtained in our experiments. As was noted above, this type of growth-promoting ability is not shared by kinetin.

In determining whether $G.A_3$ could antagonize the inhibition caused by IAA, it was noticed that the lengths of buds in IAA plus $G.A_3$ were very irregular (see Table 1). An analysis of the data suggested that those buds which had begun to elongate were being further promoted by the $G.A_3$ while others remained more or less fully inhibited. In other words, the simple promotion of bud growth exemplified in Figure 9 was being superimposed on a partial 'breakaway'. To avoid this phenomenon, a concentration of IAA high enough to prevent the 'breakaway' was used. Results are shown in Table 2. It will be seen that growth of buds in 10 ppm IAA was almost completely inhibited and that this inhibition was in no way overcome by $G.A_3$, although it was largely reversed by a suboptimal concentration of kinetin.

It is clear that the effects of $G.A_3$ and kinetin are entirely different in this system. Apparently $G.A_3$ acts primarily to promote the elongation of buds which have already been released from inhibition.

Table 1. Bud lengths obtained after 8 days growth in IAA plus either kinetin or gibberellic acid.

Medium	Extremes mm	Average mm	Standard Deviation
1 % sucrose	6 —15.5	11.8	2.9
3 ppm IAA + 3 ppm kinetin	4 —11.2	8.5	1.8
3 ppm IAA + 3 ppm $G.A_3$...	0.8—33.0	8.0	7.4

Table 2. *Comparison of effects of kinetin and gibberellic acid as antagonists to IAA.*
All figures are bud lengths in mm.

Initial	0.8
After 13 days: Control.....	10.3
in 10 ppm IAA	1.3
10 ppm IAA + 8 ppm kinetin	7.2
10 ppm IAA + 6 ppm gibberellic acid	2.4

E. *Experiments with entire shoots*

A few experiments have been carried out with etiolated shoots with the apex in situ. These shoots were cut off from the rest of the plant between the first and second nodes and set vertically into rooting bottles containing 10 ml of 1 per cent sucrose with or without kinetin. The solutions were changed every two days.

The results of a typical experiment are shown in Figure 10 A. It will be seen that the lateral buds of the decapitated shoots grew at a rate similar to that of the controls in previous experiments, while the buds on the shoots with the apex left on were totally inhibited. In the shoots with the apex on, but with the base dipping in 6 ppm kinetin, the buds grew to 71 per cent of the control value.

Similar results were obtained with seedlings raised in a greenhouse (Figure 10 B). In this case the shoots were severed from the parent plant just above the cotyledons and the lowermost bud was removed. In the shoots in 2 ppm kinetin the lateral buds grew to 74 per cent of the control value, and their growth rate at the end of the experiment was indeed just as rapid as that of the controls.

There are certain differences between this system, which makes use of endogenous auxin secreted at the apex, and the more artificial one in which IAA is supplied externally. In the entire shoots no 'breakaway' could be observed even after 10 days. Also, in the entire shoots in kinetin, growth of the lateral buds started later and more slowly than in the decapitated controls, whereas in the isolated stem sections (*cf.* Figure 4) the buds treated with IAA plus kinetin grew as fast as the controls. This might be due to the fact that in the vertical system kinetin had to be transported up the shoot, while in the horizontal shoots the buds were bathed on all sides by the kinetin. A few experiments with intact rooted plants, to which kinetin was applied at a point below a lateral bud, have indicated also that kinetin is transported only with difficulty up or down a long stem. Such local application of kinetin on intact plants was indeed found to cause marked swellings, involving both cell

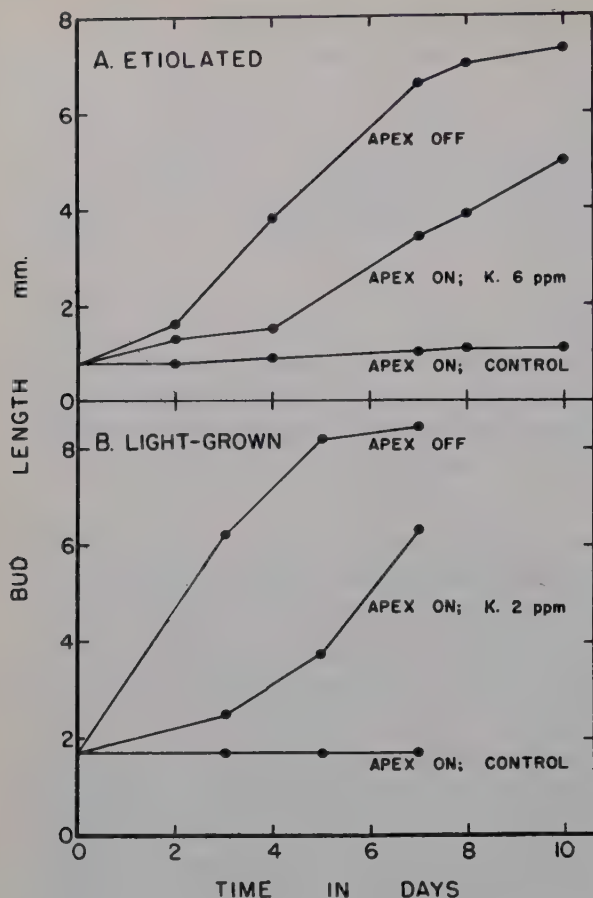


Figure 10. *Effect of kinetin on growth of lateral buds on pea shoots with intact apices. Basal medium 1 % sucrose.*

A. Shoots taken from etiolated plants. Experimental solution: 6 ppm kinetin.

B. Shoots taken from plants germinated in greenhouse. Experimental solution: 2 ppm kinetin.

division and cell enlargement, but these swellings were restricted to within a few mm. of the point of application.

Discussion

The results show that under favorable circumstances of auxin concentration, nutrition and time, kinetin makes it possible for as much elongation of lateral buds to occur in the presence as in the absence of auxin. This lifting of the inhibition is not due to any marked bud growth-promoting effect of the kinetin and in this respect it clearly differs from the intense elongation promotion caused by the gibberellins. In particular, experiments like those of figure 10 provide clear demonstration that laterals can grow out rapidly in presence of the uninjured apex.

These experiments as a whole strongly indicate that apical dominance may be due to an interaction or balance between auxin and a kinetin-like factor in the stem. Such a conclusion must, however, be advanced tentatively at present in the absence of the isolation of a kinin from higher plant tissue. It would, at least, lead to the obviation of some of the difficulties which attend theories of apical dominance based on the action of auxin alone.

Summary

The elongation of the second lateral bud of isolated pea stem sections, in sucrose solution and light, has been used to study the factors controlling the growth of lateral buds.

Several auxins at physiological concentrations inhibit the growth more or less completely. This inhibition occurs only if the auxin is applied immediately after decapitation. When IAA at concentrations below 8 mg. per liter is used as the auxin, bud development may begin spontaneously after about five days.

If kinetin at concentrations between 1 and 10 ppm is applied together with the auxin, the inhibition can be completely removed. In general the higher the auxin concentration the higher the kinetin concentration needed to remove the inhibition.

Kinetin causes bud development in the same way when the inhibiting auxin is being provided by the intact apex. It is suggested, therefore, that the normal phenomenon of apical dominance depends on an interaction between auxin and a kinetin-like substance in the plant.

Gibberellic acid also promotes bud elongation in this system, but reasons are given for believing that its action is exerted only after the inhibition has been released.

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Ascorbic Acid Oxidase in Germinating Lettuce Seeds and its Inhibition

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It has been previously suggested in this laboratory that the oxidative pathway of germinating lettuce changes with changing external conditions (Mayer, Poljakoff-Mayber and Appleman, 1957). The existence of two terminal oxidases has already been shown (Mayer 1954, Mayer, Poljakoff-Mayber and Appleman 1957). It was therefore of interest whether ascorbic acid oxidase was also present in the germinating seed and how the germination stimulating thiourea and germination inhibiting coumarin affected it. The effect of thiourea and coumarin on germination has been extensively studied in this laboratory (Poljakoff-Mayber, Mayer and Zacks 1957, Mayer and Evenari 1952, Mayer 1953, 1956).

In addition to the special interest of the function of ascorbic acid in the germinating seeds, its general role in plant tissue is of great interest. A great deal of contradictions exist in the published work. Mapson and Moustafa (1956) in an elegant paper have shown the ascorbic acid-ascorbic acid oxidase system, together with glutathione reductase, to be active in germinating pea seedlings, where it could be responsible for $\frac{1}{4}$ of the entire oxygen uptake. Eichenberger and Thimann (1957) on the other hand claim that in pea internodes the oxidase can at most be responsible for 20 per cent of the oxygen uptake and doubt even this amount. The location of the enzyme is also greatly disputed. Thus, both Mapson and Moustafa (1956) and Thimann and Eichenberger (1957) locate it in the cytoplasmic fraction, i.e. not precipitating at 10—20,000 g. Honda (1957) on the other hand, claims it to be present in the mitochondria, while Newcombe (1951) claims it to occur in the wall fraction.

A similar confusion also exists regarding the location of ascorbic acid in plant tissue. This has been reviewed by Chayen (1953). In lettuce seed ascorbic acid has been shown to be present in small amounts by Kamson-Rappaport (1957). The Italian workers Marrè and collaborators assign a special function to ascorbic acid oxidase. They have found indole acetic acid (IAA) inhibition of the oxidase. They relate this inhibition to the function and mode of action of IAA (Tonzig and Marrè 1955, Marrè, Arrigoni & Forti 1957, Marrè and Arrigoni 1957). It was therefore decided to investigate the ascorbic acid oxidase status of germinating lettuce seeds in the light of the previous work on this enzyme. The effect of thiourea has already been briefly recorded previously (Mayer 1957). In addition, the effect of changing external factors on the enzyme was also investigated. The results will be given in the following.

Methods

Lettuce seed, variety Grand Rapids, obtained from the Ferry Morse seed company, were used throughout the experiments. They were germinated at 26°C in the dark in water or test solution. All results are related to initial weight of air dry seeds. Extracts of the seeds were prepared. Early investigations showed that the enzyme could be readily extracted into water but that the activity so obtained was variable. Buffer solutions were therefore tried. Extraction into phosphate-citrate buffer markedly depressed activity, although its addition to the extract prepared in water had no effect. Grinding in acetate buffer (N/10) gave good and reproduceable extraction. The following general procedure was therefore adopted. Seeds or seedlings were ground with acetate buffer, filtered through cheese cloth and centrifuged for 5 minutes at 750 g. The extracts was then diluted with an equal volume of phosphate-citrate buffer and used for estimation of activity. The concentration of the extracts was 200–400 mg original weight of seeds/20 ml final volume of extract. When mitochondria were precipitated, acetate sucrose buffer of various molarities for sucrose was used. All the operations were carried out in a cold room at 2°C.

Ascorbic acid oxidase was determined in one of the two ways: The ascorbic acid remaining in the solution was titrated directly with chlorophenol indophenol after stopping the reaction with metaphosphoric acid. In cases where rates were determined, or thiourea, which interferes with the titration, was used, conventional Warburg techniques were applied. In this case ascorbic acid was contained in the side arm enzyme extract and suitable addition in the main compartment and the KOH in the centre well. The acid was tipped in after equilibration of 15 minutes. The extracts were not normally stabilized with gelatin. Ascorbic acid solutions were frequently prepared freshly, kept in the cold and dark and stabilized by the addition of metaphosphoric acid to a final concentration of 0.1 per cent. In all cases ascorbic acid oxidase activity was determined at 26°C. When the titration method was used, an extract equivalent to 50 mg original seeds was incubated with two milligrams of ascorbic acid under constant slow shaking in a water bath. In the manometric method generally extract equivalent to 20 mg of seeds was incubated with one mg of ascorbic

acid. The two methods did not entirely correspond. This problem will be further mentioned under "results".

In the titration technique boiled samples were used as a control, as a small amount of ascorbic acid was sometimes absorbed on the protein precipitate. In the Warburg method no such controls were needed. The endogenous oxygen uptake of the extracts was zero, presumably due to their great dilution.

Enzyme activity was completely destroyed by boiling.

Fractionation of the enzyme was attempted. The enzyme was, however, found to be extremely labile. Both acetone precipitation and ammonium sulphate fractionation, which are regarded as standard procedures, inactivated the enzyme completely. The enzyme in lettuce seed and seedlings is apparently exceptionally unstable and further attempts at fractionation were not made.

Results

The location of the enzyme was established. The initial acetate buffer extract was centrifuged at various speeds and supernatant and residue examined. When centrifugation at high speeds was used original extraction was made in sucrose-acetate buffer 0.4 *M* towards sucrose, the acetate being kept at *N*/10. The results are shown in table 1. It is seen quite clearly that the activity is predominantly in the cytoplasmic fraction. Moreover, fractionation in sucrose acetate buffer causes a certain loss of activity, as compared with activity before high speed centrifugation, while fractionation in acetate causes a slight gain in activity in the cytoplasmic fraction.

It was next determined how ascorbic acid oxidase activity changes with increasing time of germination. In addition, the effect of various treatments on the seeds during germination was determined. In each case all four treatments, *i.e.* dry seeds and those germinated for 24, 48, and 72 hours were carried out simultaneously.

The change in activity in seeds germinated in water is given in Table 2.

Table 1. *The localisation of ascorbic acid oxidase in cell extracts on centrifugation at various speeds. Results are as percentage of activity of extract centrifuged at 750 g for 5 minutes.*

Speed of centrifugation	% activity	
	Supernatant	Residue
350 g	100	0
750 g	100	0
1,250 g	100	0
1,750 g	100	0
20,000 g acetate extracted	107	10
20,000 g acetate-sucrose extracted	65	11

Activity is seen to rise sharply only after 24 hours of germination. The effect of coumarin on this change was next investigated. This experiment was carried out simultaneously with water controls. The results are given in table 3. As will be seen, the initial level of oxidase in the seeds was higher than for Table 2, but the change in coumarin and in water was identical. This variability of the behaviour of dry seeds was frequently noted and dry seeds were therefore run as a control whenever a change with time was determined. Coumarin did not in any way change the development of ascorbic acid oxidase activity in the germinating seeds, nor did it affect its activity *in vitro* at this concentration. This was further studied at different coumarin concentrations. In the range of 0—8 mg⁰/₀ there was no effect of coumarin on ascorbic acid oxidase activity, despite its effect on germination, which varied from 77—0 per cent.

The effect of thiourea on the oxidase was next studied. This has already been previously described. Seeds were germinated in 125 mg⁰/₀ thiourea and the activity of the extracts compared with those germinated in water. The activity was determined manometrically. The results are shown in Table 4. Two facts emerge: Thiourea inhibits oxidase activity completely both *in vivo* and *in vitro*. In addition, calculation of these results shows that the ascorbic acid destroyed was greater than when this was measured by direct titration, 1 mg ascorbic acid being taken as equivalent to 64 micro-litres oxygen. This difference is presumably due to the very much more rapid shaking on the Warburg apparatus and consequent greater access to oxygen, which allowed oxidation to proceed more quickly than in the titrimetric experiments. The latter were carried out in a shaking water bath with slow shaking and with liquid depth of 1—2 cm as against a few millimeters in the Warburg flasks. The trend of the curves showing the change with germination time is, however, identical. The rise in activity in water occurs after twenty four hours of germination. It was also found that there was a stoichiometric relationship between oxygen uptake and ascorbic acid destroyed, the oxygen required for complete oxidation being the theoretical one. This is in contrast to the results of Eichenberger and Thimann (1957) who claim that ascorbic acid was oxidised beyond dehydro-ascorbic acid. The inhibition caused by thiourea was not removed either by washing the seeds thoroughly, in order to remove any possible adhering thiourea, or by dialysis of the extracts. The effect of thiourea concentration *in vitro* on oxidase activity is shown in Table 5. It has been shown elsewhere that ascorbic acid affects the germination stimulation of thiourea (Mayer, Poljakoff-Mayber and Zacks, 1957). It was therefore of interest to see whether ascorbic acid could affect the inhibition of the oxidase when given together with thiourea *in vivo* and also whether ascorbic acid itself caused any change in the ascorbic acid oxidase status, of the seeds.

Table 2. *Change of ascorbic acid oxidase activity with increasing germination time for seeds germinated in water.*

Time of germination	Mg. ascorbic acid destroyed by extract of 100 mg. seeds in 15 min.
0	0.86
24	1.12
48	3.06
72	3.44

Table 3. *Change of ascorbic acid oxidase activity in seeds germinated in water and in coumarin (mg/o) with time of germination.*

Germination	Mg. ascorbic acid destroyed by 100 mg. seed in 15 min.			
	Water	% germination	Coumarin	% germination
Dry seed	2.18	—	2.18 ¹	—
24 hours.....	2.24	11	1.84	0
48 „	2.68	45	2.64	11
72 „	3.46	50	3.36	22

¹ Coumarin was added to the reaction mixture in vitro to a final concentration of 4 mg/o.

Table 4. *Oxygen uptake of extracts of seeds germinated in water or 125 mg/o thiourea for various times. The results are given as $\mu\text{l O}_2/100 \text{ mg. 15 min.}$*

Germination	Water germination		Thiourea germination	
	$\mu\text{l O}_2$	Mg. as. acid (calc.)	$\mu\text{l O}_2$	Mg. as. acid (calc.)
Dry seed	107	1.67	0 ¹	0
24 hours.....	117	1.84	0	0
48 „	272	4.35	0	0
72 „	286	4.47	0	0

¹ Thiourea added in vitro to the reaction mixture to a final concentration of 125 mg/o.

Table 5. *Effect of thiourea concentration on ascorbic acid oxidase activity in lettuce seed extracts.*

Thiourea conc. M	% inhibition of oxygen uptake
0	0
0.54×10^{-5}	7
2.8×10^{-5}	75
1.4×10^{-4}	100

Table 6. *Effect of IAA on ascorbic acid oxidase activity of extracts of lettuce seeds germinated for 48 hours in water.*

Time in min.	$\mu\text{l O}_2$ uptake by 100 mg. seeds	
	Control	IAA $5 \times 10^{-5} M$
3	77.8	73.5
6	181	167
9	270	244
12	283	278
15	288	280

Table 7. *Effect of light stimulus given to the seeds during germination on the development of ascorbic acid oxidase activity.*

Time of germination hours	Mg. ascorbic acid destroyed by 100 mg. seeds in 15 min.
0	0.76
24	1.14
48	2.00
72	2.20

Ascorbic acid did not affect the inhibition by thiourea in any way, complete inhibition being observed. When ascorbic acid was given alone there was some slight indication that it raised the ascorbic acid oxidase level of the seeds but this could not be established statistically.

In view of the results of Marrè *et al.* 1957, it was decided to test whether in lettuce seedlings also IAA would inhibit ascorbic acid oxidase *in vitro*. Some typical results are given in table 6. It is quite clear that IAA does inhibit the rate of ascorbic acid oxidation as compared to controls.

In view of the germination stimulation caused by light, the effect of giving the seeds a light stimulus during germination was tested. The seeds were given 5 mins of white light of 250 f.c. two hours after placing them in water and then germinated for various periods. Ascorbic acid oxidase activity was then determined. The light stimulus caused 100 per cent germination after forty-eight hours. The development of ascorbic acid oxidase activity was however in no way affected. Table 7 again shows that the rise in oxidase activity occurs after twenty-four hours.

Discussion

From the results obtained it will be seen that as in pea seedlings and pea internodes, the ascorbic acid oxidase of lettuce is situated in that part of the cytoplasm which is not precipitated by 20,000 g. (Table 1). The enzyme was

unstable, being inactivated by ammonium sulphate, acetone, extraction into phosphate-citrate buffer and by dialysis.

Dry lettuce seeds have an extremely high ascorbic acid oxidase activity. The amount of enzyme present in the dry seeds is adequate to mediate the oxygen uptake of the germinating seeds for the first 48 hours of germination. This calculation is based on the figures on oxygen uptake of entire seeds and seedling as given by Evenari, Neumann and Klein (1955) who found an endogenous oxygen uptake by seedlings after 30 hours of germination to be about 100 micro litres per 100 mg seeds/hour, as compared with 468 $\mu\text{l}/100\text{ mg/hour}$ here (Table 4), with ascorbic acid added. The amount of enzyme in the germinating seeds does not rise during the first twenty-four hours of germination, but then rises sharply for twenty-four hours and levels of up to seventy-two hours (Tables 2 and 4). This is paralleled by the seeds germinated in coumarin, despite the fact that the germination percentage in the latter is very low (Table 3), and by seeds germinated with a light stimulus which have a high percentage of germination (Table 7). Coumarin also did not effect the ascorbic acid oxidase activity in vitro (Table 3). Thiourea, on the other hand, was found to completely inhibit ascorbic acid oxidase both in vitro and in vivo (Table 4). This inhibition was not reversed by either dialysis or by germinating the seeds in the presence of ascorbic acid. Thiourea is known to enter the seeds quite rapidly (Mayer 1956). The amounts entering would be entirely sufficient to explain inhibition of activity in view of the low concentration of thiourea needed to inhibit the oxidase, $1.4 \times 10^{-4} M$ causing complete inhibition. Despite this fact, thiourea stimulates germination. Moreover, ascorbic acid has recently been shown to enhance the stimulation of thiourea by real interaction (Mayer, Poljakoff-Mayber and Zacks 1957). Thus it must be assumed that the ascorbic acid can affect germination quite independently of the presence or absence of its oxidase. This is of interest especially in view of the claims of Tonzig and Marrè (1955) that IAA acts through the ascorbic acid oxidase. It has been shown that IAA can affect growth of lettuce seedlings in the presence of thiourea (Poljakoff-Mayber, Mayer and Zacks, 1957). The thiourea concentration used was enough to completely inhibit the oxidase system. The effect of IAA on ascorbic acid oxidase could also be shown to be clearly present in vitro on the ascorbic acid oxidase of lettuce seedlings. This is however on the rate of oxidation by the oxidase (Table 6). It seems doubtful therefore that this IAA effect can be the basis of the action of the growth hormone in plant. Equally it seems difficult to believe that the only function of ascorbic acid oxidase is to regulate the ratio of SS/SH groups in the tissue. The amounts of ascorbic acid present in lettuce seeds and seedlings are very small (Kamsun-Rappaport, 1957) clearly suggesting a catalytic function. Moreover, although coumarin markedly affects the ascorbic acid content of

the seedlings (Kamsun-Rappaport 1957) it does not effect their oxidase (Table 3).

From the above it can be concluded that ascorbic acid oxidase can be a terminal oxidase present in lettuce seeds and seedlings. That it does so function is suggested by its very high initial content of the dry seeds. As germination proceeds other terminal oxidases become more and more active (Mayer, 1954; Mayer, Poljakoff-Mayber and Appleman, 1957) and may well replace the ascorbic acid oxidase. On the other hand, thiourea presumably at once causes a shift to some other terminal oxidase. In how far its germination stimulation can be ascribed to this action cannot be concluded from these results.

Summary

It is shown that lettuce seeds and seedlings contain large amounts of a very active ascorbic acid oxidase, which is localised in the cytoplasmic fraction of the cells. The ascorbic acid oxidase activity rises sharply after 24 hours of germination, independently of the germination percentage. The oxidase activity is not affected in vitro or in vivo by coumarin. Thiourea completely inhibits ascorbic acid oxidase in vitro and in vivo. IAA somewhat inhibits ascorbic acid oxidase in vitro. The possible significance of ascorbic acid oxidase in the germinating seeds is discussed and it is shown that it could mediate the entire oxygen uptake of the seeds. The action of thiourea on germination appears to be that of causing a change in the metabolism of the seeds at a very early stage, towards other terminal oxidases and presumably changing other aspects of metabolism also. It is shown that the action of IAA cannot be attributed solely to its effect on ascorbic acid oxidase.

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Some Further Investigations on the Oxidative Systems of Germinating Lettuce Seeds

By

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The respiration of lettuce seeds of the light insensitive variety Progress, was investigated by Levari (1). Also the oxidative properties of particles isolated from seeds of the same variety were investigated (2). From these investigations it appears that the respiration of the seeds increases during the first hours of imbibition but only to a certain level. It remains more or less steady at this level until approximately the 16th hour, when germination actually becomes evident, *i.e.* the rootlets penetrate the seed coat. From this time on, the respiration increases with increased growth of the seedlings. These investigations were continued only during the first 30 hours.

The particles isolated from these seeds were able to oxidize all the substrates of the tricarboxylic acid cycle. They showed a marked preference for malate. Their ability to oxidize glycollate and formate indicated the existence of malic shunt.

When similar investigations were carried out with another variety of lettuce, the light sensitive Grand Rapids — the results were somewhat different. The course of respiration of the whole seeds during the first 30 hours of germination was very similar (see figure 1) to that of Progress. The oxygen absorption and the CO_2 evolution, however, both were much lower in Grand Rapids than in Progress. The oxidative activity did not sediment with the particles (3), until 48–72 hours of incubation. After a period of 48–72 hours, germination was already over and growth of the seedlings was well under way. There were, however, two differences in the method of isolation of the particles in the two cases. The particles from Progress were isolated into a

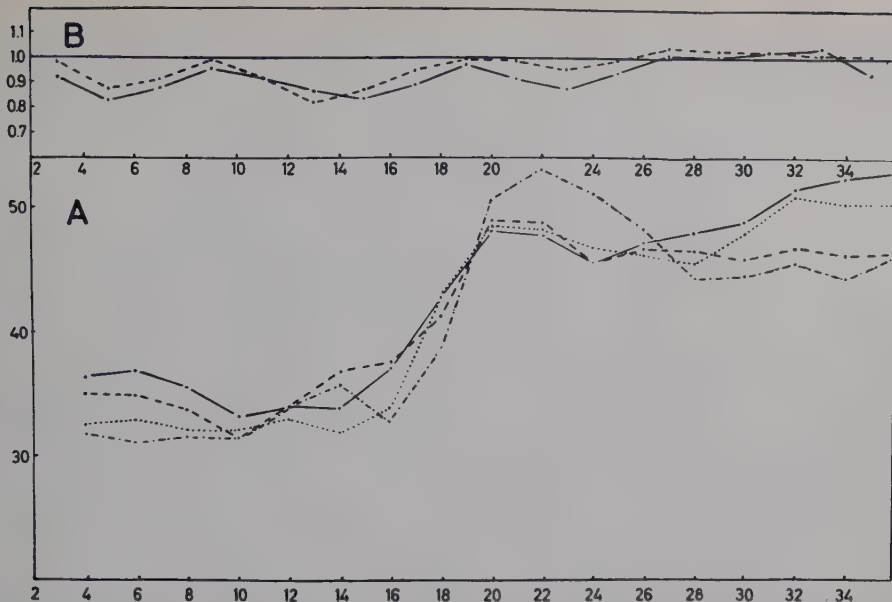


Figure 1. *The respiration of G.R. lettuce seeds germinated in water and thiourea of the concentrations of 125 mg⁰/o in the dark at 26°C. A. Oxygen and carbon dioxide curves represented as moving averages of four successive hourly readings. B. R.Q. curves for the same experiments. On the abscissa germination time in hrs., on the ordinate $\mu\text{l. gas}/100 \text{ mg. seed weight}$. A: —·—·—· O_2 water, ···· CO_2 water, ———— O_2 thiourea, —·—·—· CO_2 thiourea. B: —·—·—· water, ···· thiourea.*

medium of a higher tonicity and the centrifugation speed was higher. It was therefore decided, that for full comparison the whole work with Grand Rapids had to be repeated using the methods previously applied to the Progress seeds.

Material and Methods

Lettuce seeds of the light sensitive variety Grand Rapids were used throughout the experiments. The germination of the seeds was carried out as previously described (2). When a light stimulus was given, 5 minutes of illumination from an ordinary electric bulb were given after 2 hours of imbibition.

The particulate fraction was prepared as previously described (2). The isolation medium contained 0.1 M phosphate buffer, 0.4 M sucrose and 0.001 M MgSO_4 . The particles were sedimented at $22,000\times g$ for 20 minutes.

The oxygen consumption was determined using the conventional Warburg technique at 30°C.

The respiration of the whole seeds was determined as previously described at 26°C (4). 100 mg of seeds and 0.25 ml of fluid were used. The thiourea solution used was of the concentration of 125 mg per cent.

Nitrogen was estimated by Nesslerisation.

Table 1. *Oxidation of various substrates by particles from lettuce seeds germinated for various periods of time in light and darkness.* Reaction mixture contains: Sucrose 0.2 M; Phosphate 5×10^{-2} M; MgSO_4 2×10^{-3} M. A.T.P. 10^{-3} M; MnCl_2 0.5×10^{-3} M; Substrate 2×10^{-2} M. Particulate suspension 0.5 ml. The results are given as oxygen uptake in $\mu\text{O}_2/\text{mgN.hr.}$ and are corrected by subtraction of the endogenous respiration.

Substrate	Seeds germinated in the dark					Seeds germinated with light stimulus				
	Dry seeds	24 hours	48 hours	72 hours	96 hours	Dry Seeds	24 hours	48 hours	72 hours	96 hours
Succinate	< 5.0	< 5.0	16.0	26.0	23.0	< 5.0	9.0	10.0	34.0	52.0
α .K.G.	< 5.0	< 5.0	7.0	19.0	21.0	< 5.0	6.0	5.0	12.0	40.0
Citrate	< 5.0	< 5.0	< 5.0	5.0	6.0	< 5.0	6.0	5.0	8.0	30.0
Malate	< 5.0	< 5.0	< 5.0	5.0	26.0	< 5.0	5.0	5.0	35.0	70.0
Fumarate	< 5.0	< 5.0	< 5.0	5.0	8.0	< 5.0	< 5.0	< 5.0	29.0	56.0
Pyruvate	< 5.0	< 5.0	< 5.0	5.0	10.0	< 5.0	< 5.0	< 5.0	11.0	20.0
Endogenous	< 5.0	< 5.0	30.0	25.0	80.0	< 5.0	10.0	5.0	79.0	130.0

Results

The respiration of the whole lettuce seeds, Variety Grand Rapids (G.R.), was measured, when the seeds were germinated in water or thiourea. The results are summarized in Figure 1. As it is seen from Figure 1 the respiration remains more or less at the same level until germination proper takes place. From then on there is an increase of respiration with the increase of size, first of the embryo and then of the seedlings.

However, the behaviour of the particles isolated from G.R. varied considerably from the behaviour of particles isolated from Progress. The oxidative activity of particles isolated from G.R. seeds germinated in water in the dark and with a light stimulus is summarized in Table 1. As it is seen from Table 1, the oxidative activity of these particles is very low. It rises considerably only in particles isolated from seedlings after considerable growth (72 and 96 hours), but not during germination. Even then the oxidative activity of the particles isolated from G.R. seeds is about $\frac{1}{3}$ of the activity of particles isolated from Progress. There was no preferential oxidation of malate except in particles of seeds germinated for 96 hours in the light.

The ability to oxidize tricarboxylic acid cycle substrates develops gradually as growth proceeds. Growth proceeds more rapidly in seeds which received a light stimulus. The ability to oxidize the tricarboxylic cycle substrates also develops more rapidly in such seeds. The first enzyme system which develops appears to be the succinic oxidase one, the proper tricarboxylic acid cycle enzymes follow.

There is a difference in behaviour of particles from light and dark germinated seeds towards citrate. The ability to oxidize citrate is also affected

Table 2. *Oxidative activity of particles prepared from G.R. seeds germinated in the light.* Reaction mixture as in Table 1 but the sucrose concentration only 0.1 M. The results are in $\mu\text{O}_2/\text{mgN.hrs}$ and are corrected by subtraction of endogenous respiration.

Substrate	Germination time	
	48 hours	72 hours
Succinate	11.0	43.0
α .K.G.	10.0	50.0
Citrate	0.0	0.0
Malate	42.0	51.0
Fumarate	18.0	18.0
Pyruvate	6.0	0.0
Endogenous	68.0	185.0

by the tonicity of the isolation medium (Table 2). A lack of ability to oxidize citrate has already been reported from particles from seeds variety Progress (2). This lack of oxidation of citrate may be due to the instability of the cofactors required for the oxidation, as was suggested by Plaut and Plaut (9). This problem is being further investigated.

Lowering the tonicity of the reaction mixture increased the oxidative activity and brought about the preferential oxidation of malate (Table 2) in seeds germinated for 72 hrs. The malate oxidation was however still much lower then in seeds variety Progress.

The possibility was considered that the low activity of the isolated particles may be due either to the small amount of the enzymes themselves, or to lack of some co-factors. Addition of DPN, TPN, cytochrome C and various combinations of them did not increase the oxidative activity of the particles, towards malate. Particles from dry seeds and seeds germinated for 24 & 48 hrs were tested.

A more detailed fractionation of the homogenate was then attempted. Seeds were germinated in water for 48 and 72 hours. The complete seedlings were harvested,

Table 3. *Oxygen uptake and nitrogen content of fractions isolated by various centrifugation speeds.* Reaction mixture for oxygen uptake as in table 1 — substrate malate. The results are given as percent of the total oxygen uptake or of the total nitrogen content.

Fraction	Oxygen uptake %		Nitrogen content %	
	48 hours germination	72 hours germination	48 hours germination	72 hours germination
I	44	5.0	18.5	15.0
II	27	0.0	10.2	7.6
III	29	41.0	21.0	16.0
IV	0	35.0	2.9	4.0
V	0	19.0	47.4	57.4

Table 4. *Oxidative activity of particles isolated from G.R. seeds germinated in thiourea 125 mg%/o for various periods of time. Reaction mixture as in table 1. Results as $\mu\text{O}_2/\text{mgN.hr.}$ and are corrected by subtraction of endogenous respiration.*

Substrate	Germination time	
	14 hours	48 hours
Succinate	20.0	47.0
α .K.G.	13.0	24.0
Citrate	8.0	16.0
Malate	10.0	19.0
Fumarate	10.0	20.0
Pyruvate	< 5.0	17.0
Endogenous	10.0	-18.0

and homogenized in a mortar with the extraction medium, but without sand. The resulting brei was squeezed through cheese cloth and the homogenate was fractionated by centrifugation. The first centrifugation was done at $500\times g$ and the sediment designated fraction I. The supernatant was then centrifuged at $5,000\times g$. A compact fatty pellet was floating on the supernatant. This pellet was fraction II. The sediment was fraction III. The supernatant was centrifuged again, this time at $22,000\times g$. The sediment formed fraction IV and the supernatant fraction V. All the fractions except V, were suspended in equal amounts of sucrose buffer and their oxidative activity per mg. nitrogen towards malate was measured.

Table 3 gives the oxygen uptake of each fraction as percentage of the total oxygen uptake of all the fractions. The nitrogen content of each fraction as percent of the total nitrogen content of the homogenate is given in the same table. It can be seen from Table 3 that the oxidative activity and the enzymatic content of the lighter particles increases as growth proceeds. This can be concluded from a comparison of the oxidative activity and the nitrogen content of the various fractions isolated from seedlings of different age.

The absolute oxidative activity per mg Nitrogen of fractions III and IV increased considerably with germination time.

Germination of the seeds in thiourea stimulates the germination. The oxidative activity of particles isolated from such thiourea germinated seeds is markedly higher than that of water germinated seeds (Table 4).

Discussion

It may be concluded from these results that the relative importance of the cyclophorase system is rather small during germination and the initial stages of growth in lettuce. The importance of this system increases however as growth proceeds. It is very probable that in three to five day old seedlings, the tricarboxylic acid cycle is the main pathway of oxidative metabolism. During

Table 5. *The comparison of oxygen consumption of the whole seeds and particles as calculated in $\mu\text{l}/\text{mgN}\cdot\text{hr}$. The results for whole seeds were calculated from references 1, 6 and 7. All the data are for seed germinated for 24 hours in the light.*

Variety	Particles	Whole seeds
Progress	Endogenous 30	25
	Succinate 60	
	α .K.G. 60	
G.R.	Endogenous 10	15
	Succinate 9	
	α .K.G. 6	

germination and the subsequent period up to 48 hours there is some other system, whose function is relatively much more important. Previous findings (3) regarding the activity of cytochrome oxidase support this supposition. It was also previously suggested that in lettuce seeds a direct oxidation of glucose via the pentose cycle is possible (8). In addition a very powerful ascorbic acid oxidase was found in G.R. seeds (5). Therefore, it is plausible that during the first 48 hours of germination other oxidative pathways and electron transfer systems are relatively more important than the tricarboxylic acid cycle and the cytochrome system. The calculation of the oxygen consumption per mg N for whole seeds as compared with the oxidative activity of mitochondria also supports this (Table 5). The respiration of whole seeds was measured without the addition of any external substrate.

As growth and development proceed, the relative importance of the cyclophorase system increases, apparently not only due to increased synthesis of cofactors, but due to a change in the relative amounts of the enzymes themselves. This may be concluded from the fact that addition of cofactors such as DPN, TPN and cytochrome did not increase the oxidative activity of the particles. The results summarized in Table 3 point to the fact that during growth and development of the seedling, storage protein turns into active enzymatic protein. Thus the nitrogen content of fraction III decreases while the oxidative activity increases, as growth proceeds. This change is apparently also accompanied by an increase in the number of particles and a decrease in their size. This is indicated by the simultaneous increase of nitrogen content and oxidative activity of fraction IV. Also the oxidative activity per mg nitrogen increased in fractions III and IV as growth of the seedlings proceeded.

If we try and compare the oxidative systems of G.R. and Progress seeds during germination and growth, the following may be said: In both varieties the relative importance of the cyclophorase system gains in importance as growth proceeds. But while in Progress this system is capable of carrying out most of the oxidative activity of the seedling from the early stages, it is not

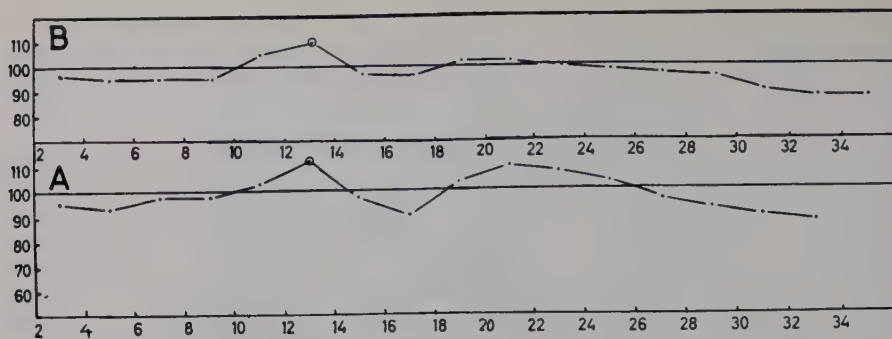


Figure 2. *Respiration of G.R. lettuce seeds germinated in thiourea (125 mg⁰/o) as per cent of their respiration when germinated in water in the dark at 26°C. The ringed points differ from the water controls at the level of 2 per cent. On the abscissa germination time in hrs., on the ordinate gas exchanged as percentage of water control.*

A CO₂ evolution, B O₂ uptake.

the case for G.R. (Table 5). In G.R. seedlings, although the relative importance of this system increases with time, it is not capable of being responsible for the oxidative activity during germination and the early stages of growth. During these initial stages in the life cycle of the G.R. some other metabolic system must be relatively more important. Although this system is not yet identified with any certainty, the possibility exists that direct glucose oxidation and ascorbic acid oxidase glutathione reductase system may serve the purpose.

Treatment of the G.R. seeds with thiourea stimulates germination, but its effect on the respiration of the whole seeds is negligible (Figures 1 and 2). As it is seen from fig. 2, the only significant changes caused by thiourea is stimulation of the gas exchange on the 13th hour of germination. Throughout the period before and after this time the change is apparently qualitative and not quantitative. Indeed, already at the 14th hour, the tricarboxylic acid cycle in the seeds germinated in thiourea was more active than in seeds germinated for 48 hours in water in the light (compare results of table 1 with those of table 4). Thiourea treatment of G.R. seeds brings about a shift in the normal metabolic sequence and the tricarboxylic acid cycle becomes active much earlier than in the water controls. In Progress however, germination in thiourea does not increase the percent germination and does not affect the oxidative activity of particles (see reference 2, figure 3). On the other hand, thiourea completely inhibits the ascorbic acid oxidase (5). Therefore stimulation of germination by treatment with thiourea is accompanied by a complete shift in the metabolic pathways and the electron transport systems in the seeds.

Summary

The oxidative capacity of the particulate fraction of lettuce seeds variety G.R. was investigated. It was shown that the particles have low activity for the tricarboxylic acid cycle intermediate. The activity increases as growth proceeds. The ability to oxidize various intermediates develops gradually with growth, succinic oxidase being the first system to develop. The oxidative capacity towards various substrates is dependent on the tonicity of the isolation medium.

It is concluded that the oxidative paths of the seeds change during germination and growth. Thiourea, which stimulates germination, does not markedly change the total gas exchange of the whole seeds. It does, however, cause a complete shift in the oxidative metabolism of the germinating seeds. This is borne out by the fact that the tricarboxylic acid cycle enzymes in thiourea germinated seeds starts operating very early during germination.

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Polyphenolase and Peroxidase in the Tomato Root Tissue and the Oxidation of Ornithine

By

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The information on the oxidation of amino acids by tissues of higher plants is still not sufficient. A direct dehydrogenase to catalyze the oxidative deamination of L-glutamic acid has been found in many plants (Bonner 1950), and a coupling oxidation system of tryptophan has been reported to occur in the tissues of pea-seedling (Wiltshire 1953). Recently Rautanen and Tager (1955) found that the mitochondria preparation from the etiolated *Avena* seedling oxidizes L-glutamic acid, L-proline and L-cysteine, either directly or indirectly. On the other hand, James and coworkers (1953) demonstrated the occurrence of ornithine dehydrogenase in the root tip tissue of tomato, *Datura*, and potato, though the detail of the result is not yet available..

The present work deals with the enzymes which appear to be responsible for the oxidation of ornithine in the tissue of the tomato root.

Material and Methods

The seeds of the tomato (ponterosa), *Lycopersicon esculentum* Mill., were soaked in water overnight and were sown in moist Petri dishes in which they germinated and grew for 4 to 6 days at 27 to 28°C in the dark, until the primary roots of the seedlings reached 3 to 5 cm. in length. These roots were excised with a sharp razor blade and submerged in 0.1 M phosphate buffer at pH 6.5. An adequate mass of this material was ground with ice-cold phosphate buffer and quartz sand in a mortar and was filtered through several layers of cheese cloth, and was cleared of debris by centrifuging for 30 minutes at 4000 r.p.m. The supernatant was used as the crude

enzyme preparation. The oxygen uptake was determined manometrically in the Warburg apparatus at 30°C. The volume of the reaction mixture was 4.0 ml. in total, and 0.2 ml. of 20 per cent potassium hydroxide was placed in the central well of the flask. The peroxidase activity was determined by the comparison of the blue colour obtained by adding benzidine and hydrogen peroxide to the enzyme, using an electric colorimeter with a filter of 660 m μ .

The absorption spectra were recorded with a spectrophotometer of the Hitachi Model, EU, using the enzyme solution as blank, and were expressed in terms of the optical density.

Results

A preliminary test in manometry indicated that ornithine is not oxidized by the enzyme and also that no effect is seen if α -ketoglutarate is added. These results may show that this enzyme extract be very weak in oxidizing the above amino acid and L-glutamic acid. Recently Wilson and coworkers (1954) demonstrated chromatographically that the particulate preparations from etiolated white lupine seedling catalyzed transamination between C¹⁴- α -ketoglutarate and ornithine. This may suggest that the oxidative process of transamination of ornithine in the tomato tissues would not be improbable. Concerning the L- and D-amino acid oxidases in microorganisms and also in animal tissues, the significance of flavine adenine dinucleotide (FAD) and flavine mononucleotide (FMN) as the coenzymes is well established (Cohen 1954). But it is not clear whether the amino acid oxidations in the plant tissues may or may not be due to these compounds.

As shown in Table 1, the addition of one of these nucleotides and methylene blue (MB) does not increase the oxygen uptake by the ornithine-tissue brei mixture. On the other hand, the oxidation of *p*-cresol and catechol, cata-

Table 1. *The effects of FAD and FMN upon the oxygen uptake in the root brei plus DL-ornithine (condition indicated in text).*

Exp. no.	Addition substances	Oxygen uptake (μ l)
I	brei (2.0 ml.) plus DL-ornithine (0.5 ml.)	50.0 in 180 min.
	brei (2.0 ml.) plus FAD (0.5 ml.)	67.0 "
	brei (2.0 ml.) plus DL-ornithine (0.5 ml.) plus MB (0.5 ml.)	42.0 "
	brei (2.0 ml.) plus DL-ornithine (0.5 ml.) plus MB (0.5 ml.) plus FAD (0.5 ml.)	43.2 "
	brei (2.0 ml.) plus DL-ornithine (0.5 ml.) plus FAD (0.5 ml.)	47.5 "
	brei (2.0 ml.) plus FMN (1.0 ml.)	57.0 in 120 min.
II	brei (2.0 ml.) plus DL-ornithine (1.0 ml.)	60.0 "
	brei (2.0 ml.) plus DL-ornithine (1.0 ml.) plus FMN (1.0 ml.)	54.0 "

DL-ornithine, 0.2 M; FAD, 60 per cent (2 mg. in 1.0 ml. water); FMN, (5 mg. in 1.0 ml. water); MB (methylene blue), 1 : 10,000; pH 7.2.

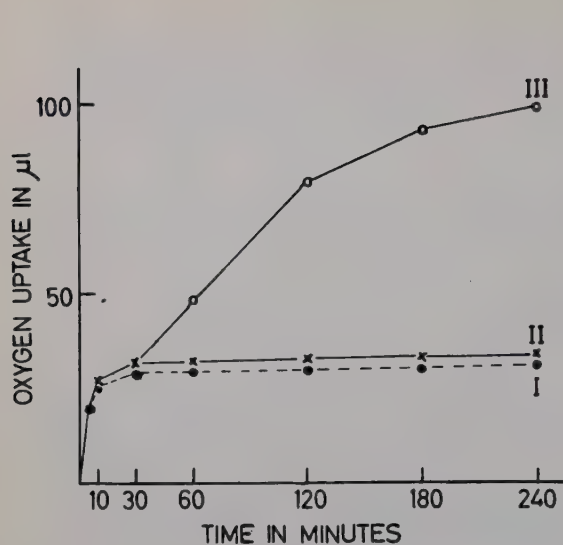


Figure 1.

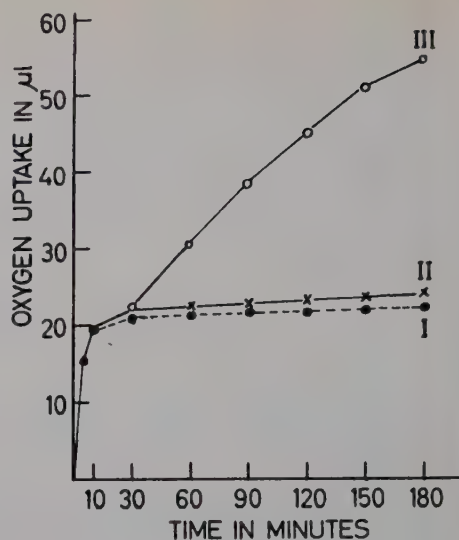


Figure 2

Figure 1 and 2. The oxidation of DL-ornithine by the purple colour pigment formed by polyphenolase-catechol complexes. Figure 1: L-Proline, figure 2: diethylamine.

Curve I: catechol with or without a secondary amine.

Curve II: catechol plus DL-ornithine.

Curve III: catechol plus DL-ornithine plus secondary amine.

The solution of the 0.02 *M* L-proline or 0.1 *M* diethylamine (pH 7.0) (each 1.0 ml.) was placed in the main chamber of the manometric flask with 1.0 ml. of enzyme solution, and 0.01 *M* catechol (1.0 ml.) was placed in the sidearm I. The sidearm II contained 0.5 ml. of DL-ornithine (0.5 *M*).

lyzed actively by the tomato enzyme, is much inhibited by phenylthiourea, sodium diethyldithiocarbamate and salicylaldoxime (Table 2). The inhibition as such is characteristic of copper enzymes, and suggests a polyphenol oxidase. In the oxidation of catechol by crude extracts of potato or mushroom

Table 2. *The effects of copper oxidase inhibitors.* Final concentration; catechol, $0.25 \times 10^{-2} M$; *p*-cresol, 0.008 *M*. Other conditions indicated in text.

Inhibitors	Final concentration (<i>M/L</i>)	Inhibition (%)	
		Catechol (in 30 min.)	<i>P</i> -cresol (in 60 min.)
Phenylthiourea	1/4 saturate	88.0	100.0
Salicylaldoxime	0.25×10^{-2}	50.0	76.0
Sodium diethyldithiocarbamate	0.25×10^{-2}	80.0	100.0

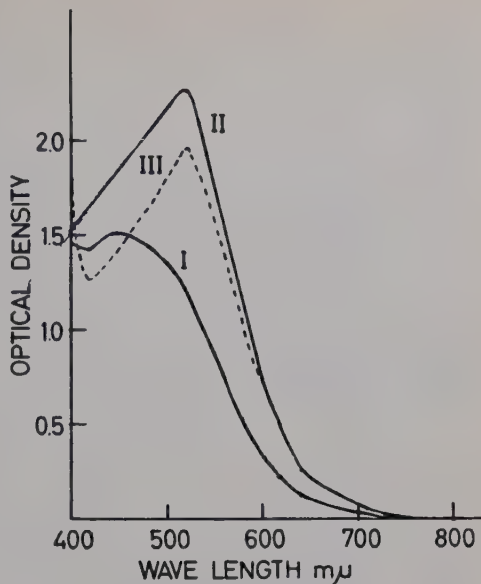
Figure 3. The absorption spectrum of the purple colour formed by polyphenolase, *p*-cresol and L-proline.

Curve I: 2.0 ml. of enzyme solution, 0.5 ml. of *p*-cresol and 1.5 ml. of distilled water.

Curve II: 2.0 ml. of enzyme solution, 0.5 ml. of *p*-cresol, 1.0 ml. of L-proline (0.02 M) and 0.5 ml. of dist. water.

Curve III: 2.0 ml. of enzyme solution, 0.5 ml. of *p*-cresol, 1.0 ml. of L-proline (0.02 M) and 0.5 ml. of L-ornithine · HCl (0.8×10^{-1} M).

As the blank, 2.0 ml. of enzyme solution was diluted with 2.0 ml. of distilled water. Determination carried out 5 hrs. after the start of the reaction.



a reddish purple colour appears at an early stage. Attention to this phenomenon was first paid by Szent-Györgyi (1925) who suggested the term 'tyrin' for this coloured substance and speculated its possible significance as a reversibly oxidizable hydrogen carrier. Many amino acids form this colour when accompanied by the polyphenolase which is oxidizing catechol, and the most striking in this regard is L-proline which was studied at first (Jackson and Kendal 1949).

This purple pigment, or 'tyrin', oxidizes certain amino acids. James and Beevers (1948) demonstrated the oxidation of L-ornithine in the presence of the *belladonna* enzyme, and Suzuki (1955 and 1957) showed the oxidation

Table 3. The benzidine test on the peroxidase activity of tomato root tissue.

Exp. no.	Root extract ml.	0.1 M Na-pyrophosphate (PH. 7.0) ml.	0.01 M MnSO ₄ ml.	0.14 M H ₂ O ₂ ml.	Benzidine acetate ml.	Optical density at 660 mμ (— log T)
1	1.0	1.0	0.5	—	0.2	0.125
2	1.0	1.0	—	0.2	0.2	0.300 (blue)
3	1.0	1.0	0.5	0.2	0.2	0.290 (,,)
4	1.0	1.0	—	—	0.2	0.000 (blank)

Reaction mixture diluted with distilled water to the double volume (5.8 ml.). Other conditions indicated in text.

Table 4. *The oxidation of resorcinol, p-cresol, L-tyrosine and DOPA by means of peroxidase system in tomato root tissue.*

Exp. no.	Substrates	0.14 M H ₂ O ₂ ml.	Phenylthiourea (saturate) ml.	Root extract pH 6.5 ml.	Optical density at 480 mμ (— log T)
I	a. resorcinol (1.0 ml. of 0.5 %)	1.0	—	1.0	0.075
	b. resorcinol (1.0 ml. of 0.5 %)	—	—	1.0	0.056
II	a. L-tyrosine (1.0 ml. of suspension)	1.0	1.0	2.0	0.030
	b. L-tyrosine (1.0 ml. of suspension)	—	1.0	2.0	0.010
III	a. p-cresol (1.0 ml. of 0.032 M)	1.0	1.0	2.0	0.380
	b. p-cresol (1.0 ml. of 0.032 M)	—	1.0	2.0	0.140
IV	a. DL-DOPA (1.0 ml. of 0.01 M)	1.0	1.0	2.0	0.645
	b. DL-DOPA (1.0 ml. of 0.01 M)	—	1.0	2.0	0.235

Reaction at 30°C.

of DL-ornithine in the presence of the enzyme from *Scopolia japonica*. As shown in Figures 1 and 2, the addition of L-proline or diethylamine, which by themselves will not be oxidized by the root extracts, did not increase the rate of oxidation of catechol. However, if ornithine was added to such system the rate of oxygen uptake began to rise immediately.

The light absorption curves of the purple products of the enzymic oxidation of *p*-cresol are shown in Figure 3. They are in a good agreement with the qualitative results in the enzymes from mushroom (Jackson and Kendal 1949) and *Scopolia japonica* (Suzuki 1957), as they have a peak at 515 mμ.

The blue colour, which often develops when benzidine and hydrogen peroxide are added to animal and plant tissues, is known to be due to the oxidation of benzidine by peroxidase, or to the peroxidase-like action of haem or haematin derivatives.

The test was made in a test tube with a reaction mixture which consists of 1.0 ml. of the root extract, 1.0 ml. of 0.1 M pyrophosphate solution (pH 7.0), with or without 0.5 ml. of 0.01 M MnSO₄ solution, and 0.2 ml. of 0.14 M hydrogen peroxide solution, amounting in total to 2.7 ml. by the addition of distilled water. The test tube was let stand 30 minutes at room temperature. Then benzidine acetate solution (0.2 ml. of 0.2 per cent solution in 10 per cent acetic acid solution) was added, and the intensity of the blue coloration was determined on an electric colorimeter.

As shown in Table 3, the results suggest the presence of peroxidase in the extract of tomato, but that the blue coloration is not obtained if hydrogen peroxide were not there.

It is well known that the peroxidase and the catalase resemble closely each other in their oxidizing ability. Certain works have shown that some amino acids, e.g., L-tyrosine, cystine, tryptophane, are oxidized by the peroxidase system, but these big molecules cannot be oxidized by the catalase. As in Table 4, the present work showed that resorcinol is oxidized by the root extract in the presence of hydrogen peroxide, and that p-cresol, dihydroxy-phenylalanine and L-tyrosine are also oxidized under the similar condition, while these reactions are not inhibited by phenylthiourea, the strong inhibitor of the polyphenol oxidase. The extent of the above oxidations was determined through the extent of the optical density of the products.

Discussion

Although James and coworkers (1953) suggested the ornithine dehydrogenase in the root tip tissues of the tomato, a more detailed result is not yet available. The survey of the tomato root extract revealed that it cannot oxidize DL-ornithine, and the transamination in this material could not be demonstrated through the determination of oxygen uptake in the presence of ornithine and the catalytic amount of α -ketoglutarate. On the other hand, the brei of the tomato root has no action of increasing the oxygen uptake in the presence of flavine adenine dinucleotide or flavine mononucleotide.

From the results as above, it was not possible to demonstrate the activity of the ornithine oxidase in the tomato root by means of the oxygen uptake determinations. Wilson and coworkers (1954), however, were successfully demonstrated chromatographically that the particulate preparation from the etiolated seedling of the white lupine catalyzes the transamination between α -ketoglutarate and ornithine. Probably the radioautography may prove the oxidative process in the mitochondrial preparations.

Sisakyan and coworkers (1946) have reported the occurrence of polyphenolase and peroxidase in the tissues of the tomato. It is known that, in the polyphenolase reaction, catechol is oxidized and that, if amino acids were present, a purple colour develops as the result of the combination of o-quinone and the secondary amine such as L-proline. This purple pigment also can oxidize certain amino acids.

Like in other plants (James and Beevers 1948; Suzuki 1955 and 1957), the purple pigment produced by the tomato root also oxidizes ornithine. It was also shown that certain other substances are oxidized by the peroxidase

system under suitable conditions. As is well known (Sizer 1953), the direct action of the peroxidase on the amino acids is usually restricted to L-tyrosine, tryptophane and cystine, but in the case of the peroxidase in the tomato root, dihydroxyphenylalanine also seems to be oxidized. It is not interpreted why the direct action of peroxidase is restricted as above. It is interesting that the problems on the oxidation of ornithine by the peroxidase system, the author will be discuss following report this problem.

Summary

The actions of polyphenolase and peroxidase from the extract of the tomato root tissues are investigated. A purple pigment is formed by the action of the polyphenolase on the catechol and L-proline, and it oxidizes DL-ornithine as demonstrated by the determination of oxygen uptake.

The author wishes to thank Prof. M. Shibata for his interest in this work.

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Effect of Temperature on Uptake of Water in Seeds

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The effect of temperature on the uptake of water in seeds has been studied by several authors. The work has been reviewed by Gessner (1956), Crocker and Barton (1953), and, with regard to grass seeds, by Lehmann and Aichele (1931). The majority of these studies may be reasonably objected to, *e.g.*, in several cases the temperature control was inadequate during the experiments, and in other cases the purity of the water (CO_2 -content, pH, content of ions etc.), so important for the rate of uptake, was not checked (Atkins 1909, Walter 1924). Also, it seems odd from a physiological point of view that in no case has the germination of the seeds been mentioned although the water uptake experiments were extended over periods long enough for germination to start. This omission is probably due to the conditions of the experiments having been unfavorable for germination, possibly through oxygen deficiency. The lack of interest in germination during water uptake experiments seems strange considering the importance of the interchange between the two processes in germination physiology as pointed out by Bogdanoff (1893).

In the studies mentioned the calculation of the amount of water taken up was made in a number of ways. Occasionally the calculation was made on the basis of the initial weight of the seeds (air-dried material) which is a not very well defined nor reproducible figure. In other cases the amount of water was given in percentage of the moist material present in each individual part of the experiment, the basis of the calculations thus varying with and during the process. Calculated in this way the uptake of water per unit of time (the

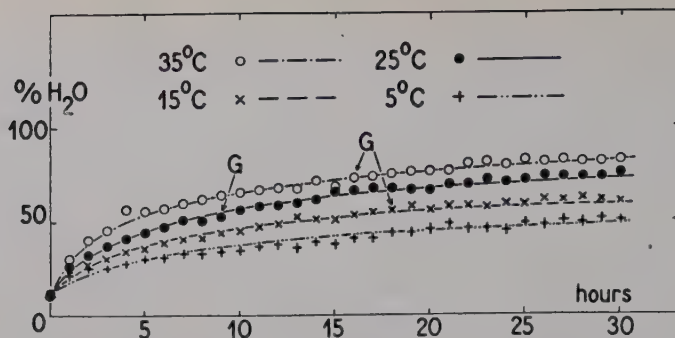


Figure 1. Uptake of water in wheat. At points marked G germination was observed. At 15 and 25° germination begins at identical water contents, approximately 55 per cent, while at a temperature of 35° no germination takes place until the water content is about 70 per cent. The water uptake processes appear to proceed more rapidly than does the process of onset of germination at this temperature.

rate) decreases with increasing uptake of water in the seeds, thus introducing the danger of misinterpretation of the results. Undoubtedly the calculation on basis of dry matter used by Stiles (1948) is the only safe one when describing imbibing processes in comparative experiments.

Material and Methods

In the present experiments the uptake of water of three representative types of seeds was determined using identical techniques at 5, 15, 25, and 35°C. In all cases the amount of water in contact with the seeds was ample and sufficiently rich in oxygen to ensure favorable conditions for germination. The purity of the water used was controlled during the experiments by measuring the conductivity and the pH. Contact with the water was established throughout the surface of the seeds, thus excluding local differences in the permeability of the seed coat (Krauss 1933, Schander 1934, Tharp 1935). Seeds of the following species were used: 1) Wheat (Svalöf Skandia III, crop 1953) representing seeds rich in starch and poor in protein and fats; 2) Pea (Dippes Maj, crop 1953) representing seeds rich in starch and protein and poor in fats; 3) Rape (summer rape, crop 1954) representing seeds rich in fats and containing protein, but free from starch.

Approximately 2 g. of seeds, or, in the case of the peas, 15 seeds were placed in a glass tube (diameter about 14 mm., length about 170 mm.) closed at each end by means of a double layer of cotton gauze fastened with a rubber band. For each particular experiment, at each particular temperature and with each particular kind of seed, is used a total of 32 such glass tubes. Prior to the experiment the glass tubes as well as the seeds were placed in dry air at the temperature of the experiment for a minimum of 6 hours. At the beginning of the experiment the glass tubes with their contents are placed in a water bath (about 30 l. of distilled water) with a

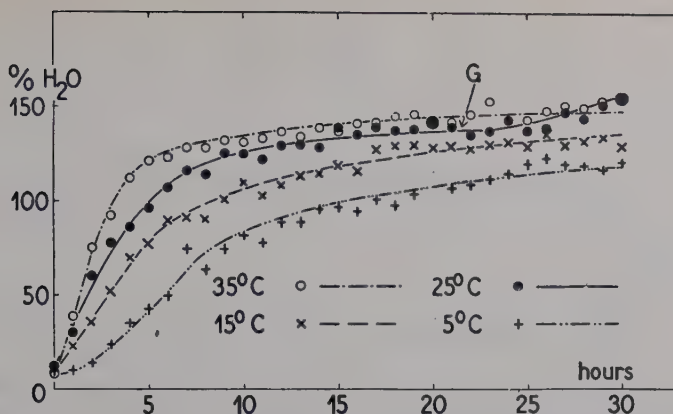


Figure 2. *Uptake of water in peas.* Germination begins at G on the 25° curve. This curve shows a distinct increase after germination corresponding to the formation of a great deal of fresh, water-containing tissue.

maximum temperature variation of $\pm 0.1^\circ\text{C}$. Five liter of CO_2 -free atmospheric air were bubbled through the water per hour, and a powerful circulation pump provided the efficient stirring of a steady stream of fresh water rich in oxygen through the tubes, past the seeds.

At the beginning of the experiments the conductivity of the water was about 1.5×10^{-5} mho, and at the end the increase was less than 5×10^{-5} mho. The pH of the water (5.5 measured by glass electrode) did not change during the experiments.

The initial water content of the seeds (at zero hour) was determined in two lots of seeds, prepared in the same way as the rest of the experimental material.

Each hour, one glass tube with seeds was taken away from the water bath. The bulk of the water was removed through one gauze end-piece by vigorous shaking. In this way the seeds were accumulated at one end of the tube, and after removal of the gauze they were shaken out onto a piece of filter paper, for wiping off the remainder of adhering water. The seeds were then weighed, and the water content was determined by drying at 100 to 105°C to constant weight, and subsequently calculated as per cent of the dry matter.

Results

The results are given in three figures (Figures 1 to 3) all showing the water content of the seeds as a function of time.

Traits common to the curves are the decrease in the rate of the water uptake throughout the experiment concurrently with the increase in water content of the seeds. This is the normal course of events and agrees well with the results of all previous experiments. Shull (1920) and Shull and Shull (1924) mathematically analyzed water uptake curves such as these and found the

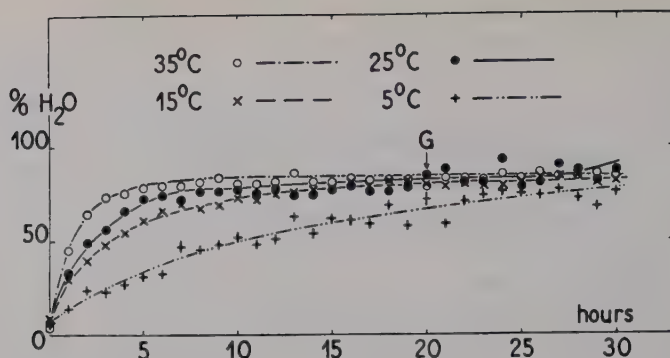


Figure 3. *Uptake of water in rape seed.* Only in these seeds the uptake seems to reach the same amount of water at all the temperatures involved. Germination takes place after 20 hours at 25° and the uptake curve for this temperature does show a tendency to increase some time afterwards.

water uptake to be an inverse logarithmic function of the amount of water already absorbed. The curve for the water uptake of the peas at 5° deviates from the course of the other curves, the rate during the first two hours being considerably lower than during subsequent time. A similar S-shaped curve for the water uptake of peas was found also by Kissler and Schmid (1932), and is probably due to the differences in the rates of uptake in seed coats and cotyledons. This difference in water uptake between the different parts of the pea may also explain the bend in the curve for 15° at a water content of about 75 per cent.

During the course of the experiments some of the seeds germinated (see the curves for peas at 25°, for rape at 25° and for wheat at 15, 25, and 35°). From the germination point onward, the water uptake in wheat proceeds at the same rate as before germination; in rape seeds, and especially in peas, the uptake rate increase after germination.

It has been shown in previous studies that the total amount of water which can be absorbed by the seed is independent of the temperature. This phenomenon is confirmed by the present results for rape seed, while the figures for peas and wheat fail to show identical curves at the end of the experiments. This may possibly be explained by a) too short experimental periods at the low temperatures, b) the fact that the seeds had started germinating and c) the above mentioned fact that in previous studies the basis of the calculations was the wet weight which might conceal differences at high percentages of water.

The final content of water found varies from species to species. The greatest one observed here was that of peas, at 140 per cent. with rape fol-

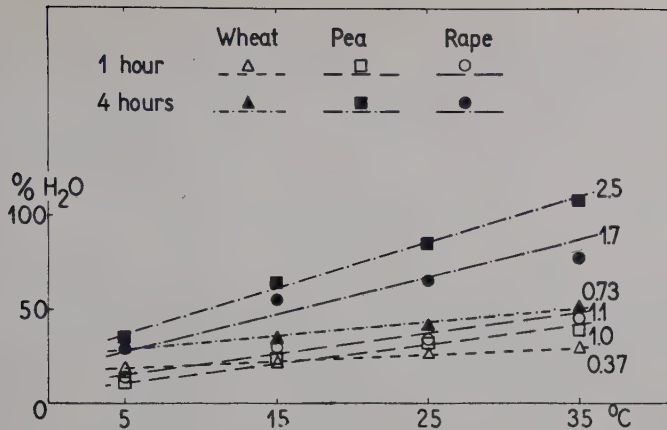


Figure 4. *The accelerating effect of temperature on the uptake of water in wheat, peas and rape after 1 hour and after 4 hours. The points represent corresponding values of temperature and water content as read from the curves of Figures 1 to 3. The figures off the curves indicate the gradient. The gradients of the 4-hours curves are considerably steeper than those of the 1-hour ones, but the corresponding gradients have not been increased to the same extent. Thus the effect of temperature is different at different times and for seeds of different species of plants.*

lowing at about 80 per cent and wheat at about 60 per cent. The sequence seems to indicate that seeds with a high protein content will take up considerably more water (and at a higher rate) than will seeds which contain mainly starch, even in cases where the protein is found together with large quantities of fats.

A closer examination of the course of the curves (see Figure 4) shows the effect of the temperature on the rate of the water uptake to vary from species to species, and also to vary with the duration of the experiment — in actual fact with the content of water — as might be expected; seeds are very far from being homogeneous, and the parts of the seeds which are still dry, and are therefore of the greatest importance for the uptake of water, change their chemical and physical properties in the course of the process. This is in disagreement with the results of Brown and Worley (1912), who claimed to have shown the rate of the water uptake to be a function of the temperature, as is the rate of chemical reactions. A thorough criticism of this work and its results is found in Shull (1920).

The temperature variation of the uptake of water might be expected to follow the change in the viscosity of the water. However, this is not the case: the effect of temperature varies as mentioned with time (content of water) and is different in the three species used. Thus it may be concluded

that the uptake of water is a question not only of the penetration of the water molecules, but is also, and maybe even more so, a question of the binding of the water in the colloids and the micellary structures of the seeds.

Summary

The uptake of water in seeds of wheat, peas, and rape was determined as a function of time at temperatures of 5, 15, 25, and 35°C. The water content of the seeds was calculated on the basis of the dry matter. The experiments were made under conditions allowing the seeds to germinate in spite of the fact that they were covered with water throughout the experiment.

The results show the rate of the water uptake to decrease with time, except in the case of peas at 5° where the curve for the uptake of water is S-shaped. A closer examination of the curves found shows the effect of temperature on the uptake of water to vary with the duration of the experiment, *i.e.*, in actual fact with the content of water. The effect of the temperature cannot be attributed to the change in the viscosity of the water.

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Factors Influencing Cell Division and Vegetative Morphogenesis of *Ophiostoma multiannulatum*

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Introduction

Previous studies of the growth of the ascomycete *Ophiostoma multiannulatum* (1, 2, 5, 6) indicated that the mode of growth, conidial, hyphal and filamentous, can be controlled by manipulation of the external environment. This present detailed study of morphogenesis was undertaken to facilitate the use of the organism in cytological and biochemical investigations which are now in progress.

The environment in which the growth of microorganisms takes place has long been known to influence the size and shape of the cells (3, 15). In many organisms complex life cycles are found which give rise to morphologically complex structures such as fruiting bodies. The metabolism determining the formation of such cells is almost completely obscure, although nutritional conditions which favour the appearance of certain types of cells are known. The uncoupling of cellular division from cell growth leading to filamentous cells has been studied in both bacteria and yeasts (8, 10). Rapkine (14) has shown that —SH groups play an important role in cellular division, and Nickerson and Falcone have recently demonstrated the participation of a

specific flavoenzyme in the reduction of S-S bonds during cellular division in *Candida albicans* (12).

The present study shows that morphogenesis in *Ophiostoma multiannulatum* is influenced by specific physical and nutritional factors such as temperature of incubation, the nature of the carbon source and the presence of as yet unidentified factors produced by the cells themselves. Furthermore, the physiological and genetical status of the inoculated cells has a profound influence upon the mode of growth of a culture.

Materials and Methods

Ophiostoma multiannulatum was maintained by regular transfers on agar slants containing Difco malt extract 25 g., Difco yeast extract 0.5 g., ammonia-hydrolyzed yeast nucleic acid 1 g., agar 15 g., and water 1000 ml. In most experiments a typical wild strain, No. 51, was used, but some of the findings have been checked with other strains of the organism.

Growth experiments in liquid media were performed in 125 ml. Pyrex flasks containing 20 ml. of a synthetic medium of the following composition: carbon source (glucose if not otherwise stated) 5 g., NH_4 -tartrate 5 g., L-asparagine 1 g., KH_2PO_4 1 g., $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g., NaCl 0.1 g., CaCl_2 0.1 g., $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 4.4 mg., $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 4.1 mg., Fe-citrate 4.5 mg., citric acid 4.5 mg., inositol 10 mg., thiamin 0.1 mg., pyridoxin 0.1 mg., and distilled water 1000 ml. The carbon source was autoclaved separately and added under aseptic conditions. The flasks were incubated in constant-temperature rooms on reciprocal shakers making two double strokes per second. Further details of the methods of culture and on the availability of various carbon sources for growth are given in reference 6.

Changes in the mode of growth of the organism were followed by visual observation of the cultures during incubation and by microscopic examination of selected samples. Photomicrographs were taken with the aid of a Leitz Ortholux Microscope equipped with a variable Heine condenser.

Description of the Morphological Types of Cells of *Ophiostoma*

When cultured on solid medium *Ophiostoma* forms a mycelium consisting of branched septate hyphae with occasional conidia formed at the tips of the hyphae. When a small amount of the wild strain of the organism is transferred from agar into liquid medium, liberation of conidia occurs. Depending on the nutritional conditions, these cells will continue to grow either in the form of budding conidia or as branching hyphae. When growth conditions are unfavourable, conidial cells may also give rise to very long filamentous cells.

Hyphal growth in liquid medium is characterized by septate, branched



Figure 1. *Branched hyphal growth of Ophiostoma obtained in a liquid, synthetic medium at 20°C. (300×).*

cells of slightly varying width (Figure 1). Such hyphal cells aggregate to form large clumps, visible to the naked eye (Figure 2).

Conidial growth is recognized macroscopically by the homogeneous appearance of a liquid culture. Microscopically the cells appear as shown in Figure 3. The size and the shape of the conidia vary somewhat according to the nutritional conditions; in old cultures they are highly vacuolated. The conidia multiply by a budding process which consists of a more or less regular division into cells of comparatively similar size. Therefore, conidial cultures give growth curves which are similar to those found with yeast and

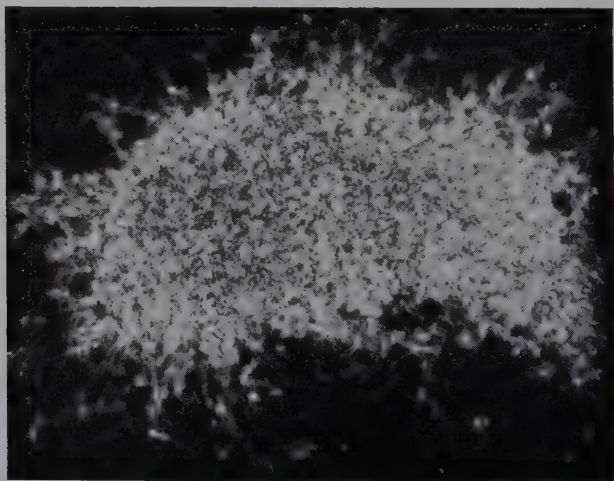


Figure 2. *Aggregated hyphal growth of Ophiostoma photographed under darkfield illumination. (200×)*



Figure 3. Budding conidia of *Ophiostoma* in a synthetic medium at 30°C. (360 \times).

bacteria. The lengths of the cells in an exponentially growing culture fall into at least three well-defined groups with peaks around 7 μ , 12 μ and 15 μ , respectively (Figure 4). One cannot, therefore, calculate an average cell length which has any meaning.

When a heavy inoculum of exponentially growing conidia is transferred into a new medium, growth starts immediately and can be followed by the change in optical density of the culture. Stable continuous cultures in the chemostat, where the amount of growth is limited by the concentration of the carbon-source in the medium, have been maintained for many days (5).

The minimal mean generation time obtained in synthetic media has been approximately three hours.

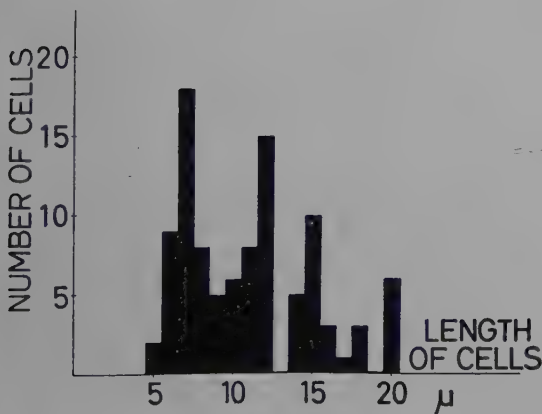


Figure 4. Size distribution of exponentially growing cells of *Ophiostoma* in a synthetic glycerol medium.

The lengths of 100 cells were measured to 1 μ , and the number of cells in each class is plotted. The distribution presented is reproducible although the exact locations of the peaks may vary somewhat.

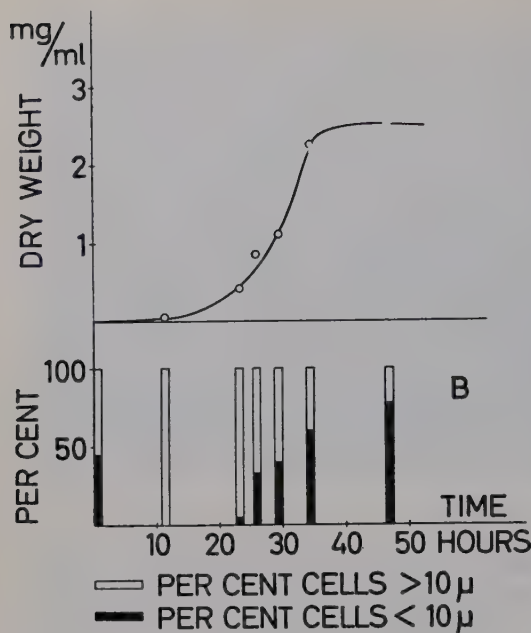


Figure 5. Growth curve of *Ophiostoma* in a synthetic glycerol medium.

A. The amount of growth.

B. The proportion between cells longer and shorter than $10\ \mu$ at various times after inoculation.

Conidia transferred from a culture where growth has been limited by the amount of carbon source do not immediately start to multiply in a fresh medium. During a lag period of up to twelve hours the cells increase in length several times. Subsequently after this period, conidia are detached and these then propagate by divisions as described above. Figure 5 illustrates a growth curve on a synthetic glycerol medium, and also the proportion between cells larger and shorter than $10\ \mu$ at different points of the growth curve.

Under unfavourable nutritional conditions very long filamentous cells showing little or no branching are obtained. Figure 6 illustrates cells from a culture where the concentration of the nitrogen source was limiting the growth. Similar cells are also obtained in the presence of certain toxic compounds.

Under many experimental conditions, cultures may contain a mixture of the various morphological types of cells. Exclusive conidial or hyphal growth is easily distinguished by visual observation of the cultures, whereas it may be difficult to estimate the amount of conidial, hyphal and filamentous growth in mixed cultures. Filtration procedures for the estimation of the proportion of conidial cells are difficult because of the tendency of the conidia to adhere to the hyphae. They can, however, give a rough estimate of the size distribution of cells in a culture.

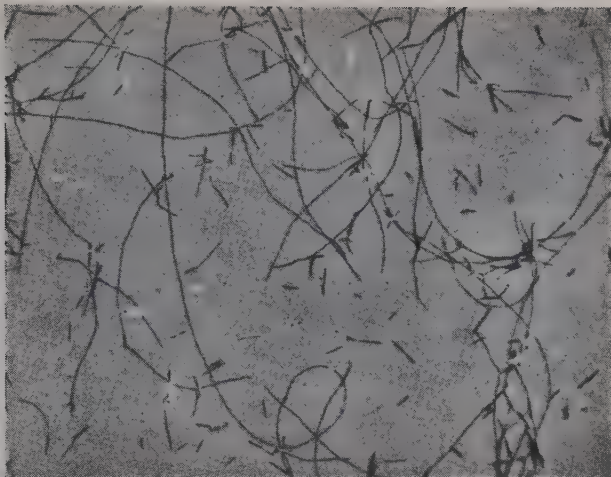


Figure 6. *Unbranched, filamentous growth of Ophiostoma in a nitrogen-deficient medium photographed with phase contrast illumination. (200 \times).*

Effect of the Nature and Amount of the Inoculum on the Mode of Growth

Mutations in *Ophiostoma*, spontaneous as well as induced by chemical or physical means, may affect the morphology of the organism. The appearance of colonies of different strains on agar plates varies with respect to colour and structure, and some auxotrophic mutants seem to be unable to grow as budding conidia in liquid media. On the other hand, Fries (personal communication) has obtained a morphological variant which is characterized by a striking increase in the formation of conidia. When grown on minimal agar, the colonies of this strain acquire a star-like appearance as a result of the formation of aerial hyphae carrying numerous conidia.

The physiological status of the inoculum is of importance in determining the growth habit of a culture. Rapid changes in the nutritional conditions usually produce a certain amount of unbranched filamentous growth before conidial or hyphal growth starts. Prolonged growth for several weeks on solid synthetic medium likewise causes a temporary loss in the ability of the organism to produce conidia in liquid media.

The number of conidia inoculated into a medium has a profound influence on the homogeneity of the eventual culture. This effect is illustrated in Figure 7. Careful washing of the inoculated cells has no significant effect on the mode of growth of a culture, and the addition of water extracts from fresh cells is also without effect. Growing *Ophiostoma* cells are known to secrete a large number of compounds such as amino acids, enzymes and ultraviolet absorbing material into the medium (6). It seems likely that con-

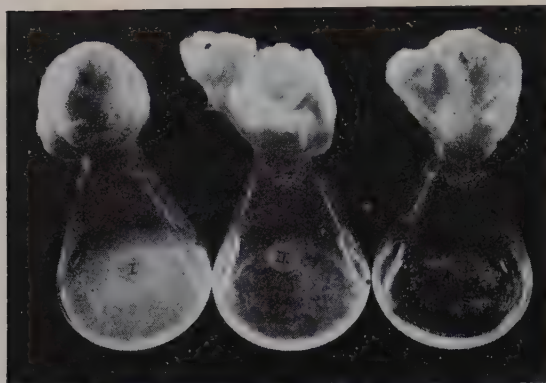


Figure 7. *Appearance of cultures of Ophiostoma in Erlenmeyer-flasks which have been shaken at 30°C for 24 hours.*

Flask I was inoculated with 10^8 , flask II with 10^7 and flask III with 10^6 cells.

dial growth is stimulated by one or several of these compounds, and that the concentration of them must reach a threshold value before regular budding occurs. If synthetic medium in which *Ophiostoma* has grown is added to fresh medium, conidial growth can be obtained even with a small inoculum. The addition of such used media to cultures also stimulates the formation of conidia by some strains which otherwise show predominant hyphal growth.

Nutritional Factors Affecting the Mode of Growth of *Ophiostoma*

As already mentioned, growth of *Ophiostoma* in deficient media often gives rise to unbranched filamentous cells. If a heavy suspension of conidia is inoculated into a nitrogen-free medium, the culture will increase in dry weight several hundred per cent, most of the cells forming long filaments, as shown in Figure 6. Poor nitrogen sources, such as inorganic ammonium salts, likewise cause a high proportion of filamentous cells.

Ophiostoma is an obligate aerobe. Standing liquid cultures grow only in the form of a thin hyphal mat on the surface of the medium. The pH of the medium does not influence the morphogenesis of *Ophiostoma* in the region where growth is permitted, probably because the cell content is protected from external pH variations. The temperature of incubation is especially important in determining the growth habit of *Ophiostoma*. Growth is obtained at all temperatures from 2°C to 30°C. At temperatures higher than 30°C only a small amount of filamentous growth is obtained. Below 20°C much of the growth is in the form of hyphae while at or near 30°C conidial growth is favoured.

The nature and the amount of the carbon source in the medium is an important factor in determining the proportion of cells growing as hyphae or as conidia. Growth with cellobiose, lactose and raffinose is slower than with glucose, fructose, mannose, saccharose and maltose, but at 25°C there is more conidial growth with the first group of compounds than with the latter. Glycerol is an excellent carbon source and stimulates the formation of conidia which are significantly smaller and more spherical than those obtained on any carbohydrate. If the concentration of the carbon source is greater than 0.5 per cent, there is a significant increase in the formation of hyphal cells. Acetate, high concentration of ethanol, and other compounds in which growth is very poor (6), cause the formation of filamentous cells.

The addition of certain compounds to the minimal synthetic medium for *Ophiostoma* results in typical changes in the mode of growth of the organism. Malt extract produces an increased production of branched hyphae. When added to give a final concentration of one per cent or more, most growth is obtained as hyphal aggregates (Figure 2). Yeast extract and extracts from lyophilized *Ophiostoma* cells likewise increase the formation of hyphae. The factor or factors stimulating hyphal growth are readily dialysable but are probably not amino acids, because the addition of hydrolyzed casein does not produce the same effect. Commercial preparations of ribonucleic acid from yeast (Nutritional Biochemicals Corp.) cause a partial change from conidial to hyphal growth in liquid cultures. This effect is strikingly increased when an alkaline hydrolysate of the preparation is used. A concentration of 0.1 mg./ml. of the hydrolysate causes almost exclusive hyphal growth. 5'-Ribonucleotides, ribonucleosides and the free purines and pyrimidines do not produce such an effect; in fact, the presence of 50 µg./ml. of adenine causes a distinct inhibition of growth resulting in nonseptate filamentous cells. More quantitative work is obviously necessary to characterize the compounds causing hyphal growth, but preliminary fractionation work indicates that the effect may be the result of a combination of several factors rather than one single compound.

Cysteine can convert certain normally filamentous strains of *Candida albicans* into the yeast phase (12), but it is without apparent effect on *Ophiostoma*. At a concentration of 10^{-2} M cysteine does not increase the formation of conidia at low temperatures and with small inocula, but rather increases the formation of unbranched filamentous cells.

Some of the compounds which have been found to inhibit the cell division of certain yeasts (15) have been tried on *Ophiostoma*. Cobalt, boric acid, penicillin, camphor, and acriflavin at various concentrations have produced very little morphological effect in *Ophiostoma*, although cytological work

could possibly demonstrate disturbed mitotic activity in the presence of some of these compounds. The phytohormones, 3-indole acetic acid and kinetin, were also without effect. The folic acid analogue aminopterin is a powerful inhibitor of the cell division of many microorganisms (10) probably through interference with nucleic acid biosynthesis. Aminopterin at a concentration of 5×10^{-6} M reduces the rate of growth of *Ophiostoma* in synthetic medium, and almost all the growth is in the form of unbranched filamentous cells.

Discussion

The various types of growth of *Ophiostoma* may be summarized in the following way.

HYPHAL GROWTH:

Branched, septate cells producing only occasional conidia at tips of cells.	In liquid media at low temperatures and on solid media. Hydrolyzed commercial yeast nucleic acid and various extracts from natural sources stimulate the formation of hyphae.
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CONIDIAL GROWTH:

Ellipsoidal or rod shaped cells multiplying by monopolar budding.	In synthetic liquid media at temperatures close to 30°C. Composition of the medium influences the size and shape of the cells.
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FILAMENTOUS GROWTH:

Nonseptate cells with no branching.	In nitrogen deficient media and during unfavourable growth conditions such as low oxygen tension and in the presence of certain compounds.
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The formation of nonseptate filamentous cells is the result of what has been termed uncoupling of cell division from cell growth. Conidial growth, on the other hand, reflects a metabolism which particularly favours cell division. Branched hyphal cells are more differentiated than either conidia or filamentous cells and probably reflect a different balance between the synthesis of structural cell material and nuclear divisions. All three types of cells may be found simultaneously in a culture, as well as some which are difficult to classify. This sometimes makes the judgment and interpretation

of the mode of growth of *Ophiostoma* difficult and subjective. In addition to a cytological investigation of the various morphological types of cells, a description of their growth in terms of protein and nucleic acid synthesis would be desirable. Furthermore, a characterization of the compounds stimulating hyphal growth requires a quantitative assay in order to distinguish between the various types of growth.

The data presented in this paper do not permit a discussion of the biochemical background of morphogenesis in *Ophiostoma*, and no attempt will be made to interpret the results in detail. It is conceivable that filamentous growth may result from either a lack of certain metabolites necessary for cell division or by a specific blocking of the mitotic process. The formation of more or less abnormal cells during conditions of slow or inhibited growth and starvation of cultures is frequently found in both bacteria and yeasts. Specific inhibition of cell division without any effect on the rate of cell growth is only obtained in the presence of a few chemicals (9) and in certain mutant strains of yeasts (11). This indicates that cell division normally is closely coupled to the formation of new cell material, and the hypothesis has been put forward that the size and shape of a cell is dependent upon the balance between the synthesis of protoplasm and the frequency of nuclear divisions (4). Any change in the nutritional conditions which influences this balance may then change the appearance of the cells, thus explaining the apparently unrelated effects of such factors as temperature of incubation and the nature of the carbon source on the size and shape of growing microorganisms. The large variations in the effect of various compounds on cell division in many microorganisms does not necessarily indicate widely different mechanisms of cell division, but may be explained by differences in the permeability of the cells.

Very little is known about the metabolism governing the differentiation and branching of fungal cells. Lilly and Barnett (7) list a number of conditions which favour the formation of fruiting bodies and spores of various organisms, but there seems to be no simple rule covering all cases. Hyphae of most strains of *Ophiostoma* detach a certain number of conidia during growth in liquid media but most of these conidia develop into new branched hyphal cells unless the conditions of growth are favourable for conidial budding. The importance of the amount of the inoculum for the maintenance of conidial growth shows the mutual growth-stimulating influence of these cells, but nothing is known about the difference in metabolism between hyphal and conidial cells.

In conclusion, *Ophiostoma* offers an interesting subject for the study of both the mechanism of cell division and problems related to the differentiation of microorganisms. In addition to giving information useful for con-

tinued physiological and biochemical studies of the organism, further details on its morphogenesis might be helpful in the interpretation of morphological phenomena found in other microorganisms.

Summary

The vegetative growth of the fungus *Ophiostoma multiannulatum* is either in the form of branched, septate hyphae or in the form of budding conidia. Under poor conditions of growth, such as nitrogen deficiency or in the presence of toxic compounds, nonseptate, filamentous cells are obtained.

Exclusive conidial growth can only be maintained in vigorously aerated liquid media incubated at about 30°C. The size and shape of the conidia are influenced by the composition of the medium and mutant strains vary in their ability to propagate as budding conidia.

Hyphal growth is favoured on solid media, in liquid media at low temperatures and by the presence of a number of extracts from natural sources. Hydrolyzed commercial yeast nucleic acid is particularly effective in stimulating hyphal growth, but no single factor responsible for this effect has been isolated.

The morphogenesis of *Ophiostoma* is briefly discussed in relation to the same type of phenomena in other organisms. Although nutritional conditions which favour the different types of growth are known, additional cytological and biochemical data are required before anything can be said about the metabolism governing the vegetative morphogenesis of the organism.

The authors are grateful for encouragement from Professor Elias Melin and for many helpful suggestions from Professor Nils Fries during the course of this investigation. This work has been aided by a grant from the Swedish Natural Science Research Council.

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Action Spectra of Phototaxis and Related Problems in Volvocales, Ulva-Gametes and Dinophyceae

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Introduction

The term phototaxis was introduced by Strasburger (1878) to distinguish the photic reaction of freely moving organisms from the phototropism of sedentary plants. Two different types of phototaxis are considered: 1) Phobophototaxis is a "shock reaction" which causes the bacteria, *e.g.* purple bacteria, to collect in the light. The phobophototactic bacteria do not have the ability to orient according to the direction of the light and to swim toward (or away from) a light source. 2) Topophototaxis is an active orientation in relation to the direction of the light and a swimming toward a light source (positive phototaxis) or away from it (negative phototaxis). In organisms showing topophototaxis, a photosensitive spot can be localized.

Since the early experiments of Cohn (1865) it has been known that some zoospores show phototaxis in blue and green light, but not in red light. Strasburger (*l.c.*) made experiments both with colored glasses and solar spectra, and concluded that the effect was on the protoplasm "as such" because color-

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less zoospores also show phototaxis. Engelmann (1882 a) reported that *Euglena viridis* in a microspectrum collected around 470 to 490 m μ , and Loeb and Maxwell (1910) showed that *Chlamydomonas* aggregated in the blue part of the spectrum.

These early experiments were of a qualitative nature; the phototactic effect of different wave-lengths was only approximately determined. When the intensity of the exciting light of different wave-lengths is known, and the phototactic activity in these wave-lengths is measured (as swimming rate, sensitivity, preference, etc.), the phototactic effect of different wave-lengths can be computed. When this effect is plotted versus wave-length, an action spectrum of phototaxis is obtained. The term, action spectrum of phototaxis, was not used in earlier experiments but in this paper it will be applied in cases when the requirements for the term are met.

Action spectra of phototaxis in topo-phototactic algae, zoospores, and gametes have been measured by different investigators using various methods. Mast (1917) recorded the orientation of organisms in a field of light consisting of two beams crossing at a right angle. One beam of known energy distribution with wave-length was from a monochromator, the other beam was composed of "white" light. At each wave-length, the "white" light was adjusted in intensity until the organisms proceeded along a fixed path. The effectiveness of the different wave-lengths was computed to obtain action spectra of phototaxis based upon the law of the force parallelogram ("Resultantgesetz"). Mast (*l.c.*) reported that the action spectra of phototaxis varied from species to species, but for all the species investigated he obtained single-peaked curves with maxima in the blue and green regions of the spectrum. Laurens and Hooker (1920) determined the relative stimulating value of light of equal radiant energy content and the relative rate of locomotion for *Volvox globator*. The action spectrum of phototaxis is represented by a single-peaked curve with a maximum at 494 m μ . Luntz (1931) used spectral lines and determined the relative sensitivity of green and colorless flagellates. The maximum sensitivity of the green flagellates was at 492 m μ , and that of the colorless form (*Chilomonas*) was at 366 m μ . Bünning and Schneiderhöhn (1956) determined the action spectra of positive and negative phototaxis in *Euglena*. They reported maxima for positive response around 490 m μ and for the negative response, around 415 m μ . Positive phototaxis was determined by the lowest light intensity which produced detectable stimulation, and the measurements of negative phototaxis were based upon intensities which caused reversal from positive to negative response.

Before the turn of the century, Engelmann (1882 a, 1882 b) performed his well known experiments on photosynthesis and phototaxis, using a *projected microspectrum*. Cohn (1865) and Strasburger (1878) also applied spectra in some of their work on phototaxis. Since these investigators did not attempt

to equalize or measure the energy along the wave-length scale, they did not obtain quantitative information on the action spectra of phototaxis and photosynthesis. Projected spectra have been applied recently in two different problems dealing with action spectra of biological processes. In analyzing the action spectrum for light-induced changes in the protoplasmic viscosity in leaves, Virgin (1954) projected upon the leaves a spectrum having an intensity gradient normal to the wave-length scale, and measured the viscosity changes. In Virgin's experiments the energy was equalized along the spectrum and the intensity gradient perpendicular to the wave-length axis was linear. The action spectrum could hence be observed directly by this procedure. Later, Babushkin (1955) used a projected spectrum in the study of the action spectrum of phototaxis in chloroplasts. The present paper will describe a somewhat similar method in the study of action spectra of topo-phototaxis in flagellates.

Methods

Manten's principles used for determining the action spectrum of phototaxis in the phobo-phototactic purple bacteria (Manten 1948, Milatz and Manten 1953), have been adapted to measuring the topo-phototaxis of flagellates as follows: when positively reacting topo-phototactic organisms in a vessel are illuminated from one side, they will swim toward the light and collect at the wall nearest the light source (Figure 1, upper left). If in addition to this light, they are also exposed to light of the same wave-length but of a higher intensity from the opposite side, they will swim to the other side and collect there (Figure 1, upper right). If the organisms are illuminated from both sides with light of equal intensity and wave-length they will, by random motion, become evenly distributed throughout the vessel (Figure 1, lower left). If the light from one side is kept constant in wave-length and intensity and used as a reference beam, the phototactic effect of light of different wave-lengths coming from the opposite side can be determined. This is done by altering the intensity of the exciting beam until the organisms are brought to the random motion stage (Figure 1, lower right).

If the intensity of the reference light is I_{ref} (in quanta/cm².sec.) and the intensity of a monochromatic light of wave-length λ_a when the organisms are balanced between the two lights is I_{λ_a} (in quanta/cm².sec.), the ratio

$$\frac{I_{\text{ref}} \text{ quanta/cm}^2.\text{sec.}}{I_{\lambda_a} \text{ quanta/cm}^2.\text{sec.}} = W_{\lambda_a}$$

is defined as the phototactic effect of light of wave-length λ_a with respect to the reference light. As the intensity and wave-length of the reference light are kept constant this gives:

$$K \cdot \frac{1}{I_{\lambda_a} \text{ quanta/cm}^2.\text{sec.}} = W_{\lambda_a}$$

where K is $I_{\text{ref}} \text{ quanta/cm}^2.\text{sec.}$ In other words, the phototactic effect is proportional to the reciprocal of the intensity causing the random motion stage. The values of

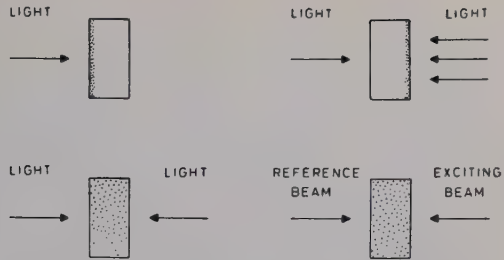


Figure 1. The distribution of topo-phototactic organisms in a vessel under different light conditions. For explanation see text p. 120.

W_λ plotted against wave-length give an action spectrum for the effectiveness of different wave-lengths for phototaxis. Manten (1948) and Milatz and Manten (1953) working on the phobo-phototactic purple bacteria showed that the action spectrum of phototaxis as defined, with the values of W_λ plotted on a linear scale, is identical with the absorbance ($\log \frac{I_0}{I}$) of the light-absorbing pigment complex involved in phototaxis. The above statement will hold true in topo-phototaxis only if certain specific requirements are met. This matter is discussed later in this paper (p. 147).

In practice, the measurements can be simplified by illumination of the vessel from one side with a reference beam uniform in intensity; and from the other side with an exciting beam having an intensity gradient (Figure 2). When the two lights are properly chosen in intensity and wave-length, the organisms will, at high intensity of the exciting beam, swim toward it and will collect in a certain region at the wall nearest the exciting light source. At lower intensity of the exciting beam, they will be more attracted by the reference beam and will collect at the wall nearest this light source. In between, there will be a certain region where the organisms will swim by random motion. When the intensity of the exciting beam and its gradient are known, W_λ can be computed from the position of the organisms showing random motion.



Figure 2.

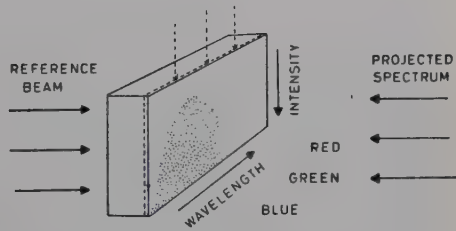


Figure 3.

Figure 2. The distribution of topo-phototactic organisms in a vessel illuminated from one side with a reference light uniform in intensity and from the other side with exciting light having an intensity gradient.

Figure 3. The measurement of the action spectrum of topo-phototaxis using a projected spectrum with an intensity gradient normal to the wave-length scale, and a reference light uniform in wave-length and intensity. Dotted lines show beam for photography or tracing of pattern. For further explanation see text p. 122.

Another method of measuring the action spectrum of phototaxis in a single exposure is to replace the monochromatic exciting beam with a projected spectrum having an intensity gradient at a right angle to the wave-length scale (Figure 3).

Under these light conditions the organisms will collect on the side of the vessel illuminated by the spectrum wherever this light is more effective than the uniform reference beam. If the spectrum is adjusted to have equal numbers of quanta along any horizontal line, and the intensity gradient at a right angle to the wave-length scale is linear from full intensity to zero, the region of random motion will form a curve representing the action spectrum of topo-phototaxis for the organism.

The organisms which have collected at the wall illuminated by the spectrum are made visible by shutting off both the reference beam and the projected spectrum, and then illuminating these organisms by intense white light from above. This was done by sending a narrow beam of light as indicated by the arrows and the dotted lines in Figure 3. Due to the Tyndall effect, the organisms show up as light spots against a dark background. This pattern can either be traced or photographed.

Apparatus

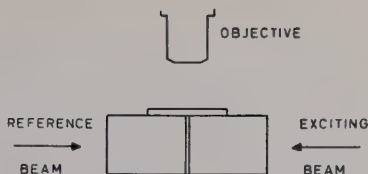
The Point-by-Point Method

The balancing of the organisms by this method was observed under a low power microscope in the vessel illustrated in Figure 4. The vessel was 22 mm. long, 12 mm. wide, and 8 mm. high. It was divided into two chambers by a vertical glass wall and partly covered, leaving space at both ends for filling. The vessel was made of pieces of microscope slide cemented together with Araldit, type XV. It was placed in a light-tight box which had windows for the reference beam, the exciting beam, and the monitoring light. The monitoring light was low intensity dark red, illuminating the vessel from below. The distribution of the organisms was observed most conveniently along the vertical glass wall which divided the vessel. Two different monochromators were used to provide the exciting light.

(1) At Hopkins Marine Station the monochromator of a Coleman spectrophotometer was used, with a band width of 5 m μ . The intensity of the exciting beam was adjusted by a variable transformer. The energy produced by a certain setting of the transformer at a given wave-length was derived from calibration curves based upon measurements by a calibrated thermopile. The intensity of the exciting beam ranged from 10 to 140 ergs/cm².sec. The wave-length of the reference beam was 405 m μ . The light source for the reference beam was a 6V, 25W microscope lamp used with a collimating lens. The light, 405 m μ in wave-length, was isolated with a combination of an interference filter, a blue glass filter, and 1 cm. of 6 per cent CuSO₄ solution. The intensity of the reference beam was 57 ergs/cm².sec. The measurements with the monochromator at Hopkins Marine Station were performed without the wedge illustrated in Figure 2.

(2) At the Carnegie Institution, the transmission grating monochromator of French *et al.* (1949) was used to produce the exciting beam. The full band width was set at 10 m μ . The intensity of the exciting beam was altered by a variable transformer, and the energy measured by a calibrated thermopile. The energy of the exciting beam ranged from 50 to 800 ergs/cm².sec., depending upon the species and the wave-length used. The light source of the reference beam was a 6V, 25W auto-

Figure 4. *The vessel used in the point-by-point method (side view).*



mobile lamp. A broad band in the blue part of the spectrum was isolated with a 4305 Corning filter. The light sources for both the exciting and reference beams were stabilized with a voltage regulator. In order to simplify the measurements as described in Figure 2, an optical wedge was inserted in the reference beam and an image of it projected by a lens system on the transverse vertical glass-wall (see Figure 4), thus producing an intensity gradient. The intensity gradient was measured with an IP22 photomultiplier tube mounted in a light-tight housing, with a 1×6 mm. rectangular window. After entering this window, the light was diffused by a piece of opal glass. The photomultiplier tube was mounted on a mechanical stage with the slit oriented perpendicular to the intensity gradient in the plane of the transverse glass-wall. Readings were taken at each millimeter interval across the reference beam. The absolute energy of the reference beam was not measured, but an intensity was selected which suitably balanced the monochromatic exciting beam.

The Method with the Projected Spectrum

Schematic diagrams of the apparatus used in the measurements of action spectra of topo-phototaxis according to the method with the projected spectrum, illustrated in Figure 3, are shown in Figures 5, 6, and 7. The unit producing the projected spectrum and the reference beam is shown in Figure 5. The light source, I_1 , was a General Electric microscope illumination lamp (18 A T10/1—6V Medium SR8 Fil.) operated at 7.5V. An image of the ribbon filament of this lamp was projected by the spherical mirror, M_1 , onto the V-shaped slit, S , mounted on a photographic wedge (detail in Figure 6). The heat was removed by the filter, F_1 . As the focal length of the spherical mirror is shorter than that of the lenses, L_2 and L_3 , the light beam was made more convergent by the lens, L_1 . After the beam had passed through L_1 , L_2 , the grating, G , and L_3 , a spectrum 4.5 cm. long and 2 cm. high was formed on the sample, Sm . The source of reference light, I_3 , was a 150W frosted bulb. The light from this lamp passed through a 4305 Corning glass filter, F_2 , and illuminated uniformly the opal glass, O . An image of the latter was focused on the sample, Sm , by the lens, L_4 . The light sources, I_1 and I_3 , were stabilized with a voltage regulator.

The spectrum was calibrated for wave-length by replacing the light source, I_1 , by the mercury lamp, I_2 , and the optical system adjusted for this light source. The mercury lines of 405, 436, 546, and 578 $m\mu$ were traced on a glass plate, P , which was placed in front of the sample, Sm , when the collection of the organisms was recorded. The width of the mercury line images at the bottom of the spectrum was 33 $m\mu$. Due to the tapered shape of the slit, this width decreased regularly from the bottom to the top of the spectrum. It was about 15 $m\mu$ at the place where the shoulder was recorded in the action spectra of phototaxis in the *Volvocales*,

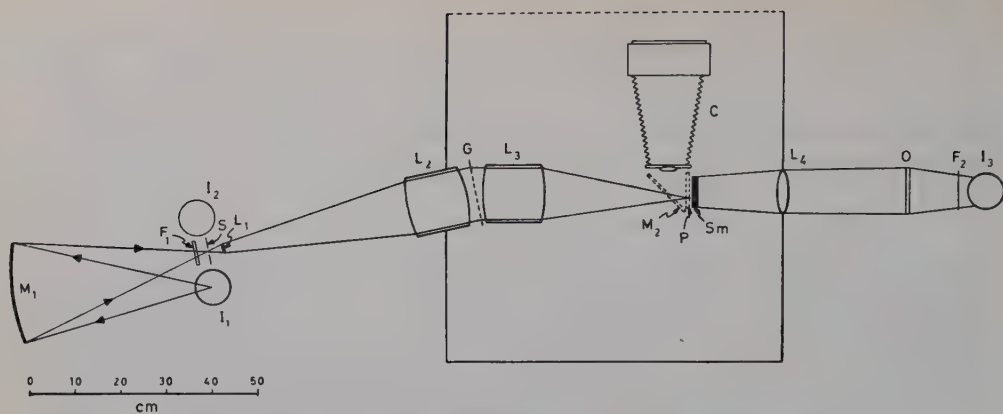


Figure 5. Schematic diagram of the unit producing the projected spectrum and the reference beam (top view). For explanation see text p. 123.

and about 10 μ where the maxima were recorded. Experiments with narrower slits did not reveal any new detail in the action spectra measured.

Results were recorded either manually by tracing on the calibrated glass plate, or by photography using a camera, C, and a removable mirror, M_2 . The organisms which had collected at the wall illuminated by the spectrum were made visible as described above (see Figure 3). A schematic diagram of the set-up for this illumination is shown in Figure 7. The light source, I_4 , was the same kind of lamp as I_1 in Figure 5. An image of the ribbon filament of this lamp was focused by the lens, L_5 , and the mirror, M_3 , on the slit, S_2 . An image of this slit was focused by the lens, L_6 , on top of the sample, Sm, from 0 to 2 mm, inside the wall illuminated by the spectrum as illustrated in Figure 3.

What should be photographed and what should be traced manually was determined by the experiment. A relatively long photographic exposure was required since only light scattered by the organisms entered the camera. An illumination of at least one minute was required even with fast film and forced developing. During this illumination by a new light source at a right angle to the first ones, the pattern of collection in most cases was distorted due to phototactic movements of the organisms. However, in many cases the organisms became attached to the wall during the experiment and stayed there even when illuminated with other lights. This was particularly true for the negatively reacting *Volvocales*. In these experiments the photographic method was used. The manual tracing was necessary when the *Volvocales* showed a positive reaction, and in all the experiments with the *Dinophyceae*. The curve representing the region of random motion was easily traced in from two to five seconds with high precision. The photographic method should have several advantages over a manual tracing. In our case it gave a direct three-times enlargement on the negative, which in turn could be enlarged further. Small details in the curve might thus be discovered. However, when the manual tracing and the photographic method were compared, the same curves were obtained.

The projected spectrum in our experiments was not corrected for equal numbers of quanta across the spectrum. The energy distribution with wave-length and the

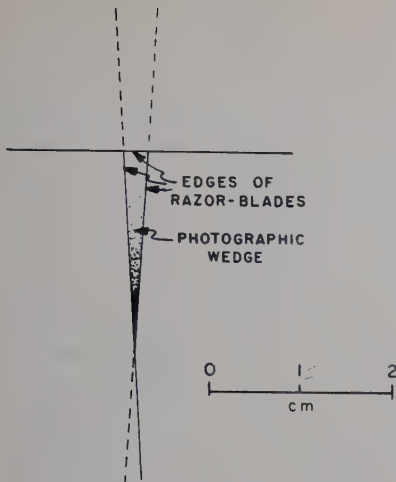


Figure 6.

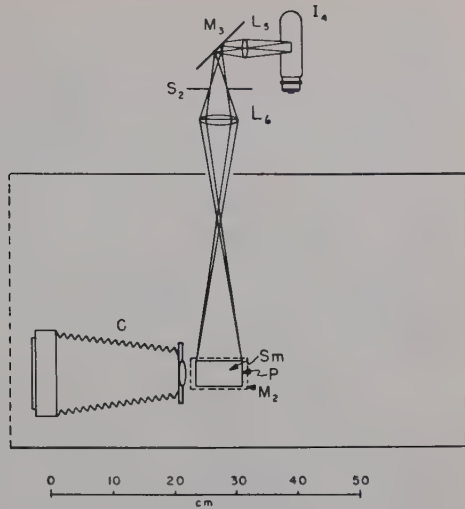


Figure 7.

Figure 6. The triangular shaped slit producing the intensity gradient of the spectrum. The photographic wedge increases the gradient produced by the tapered slit.

Figure 7. Schematic diagram of the set-up used for the illumination of the organisms collected at the wall illuminated by the spectrum (side view). For explanation see text p. 124.

intensity gradient from bottom to top of the spectrum were measured with the IP22 photomultiplier tube mounted in the housing mentioned earlier (p. 123). The sensitivity of the photomultiplier tube as a function of wave-length was determined by use of a thermopile.

In measuring the intensity of the spectrum as a function of wave-length, the slit of the housing was oriented in the plane of the spectrum normal to the wave-length scale and readings were taken at 20 mμ intervals. In measuring the intensity gradient, the slit was oriented parallel to the wave-length scale and readings were taken at 1 mm. intervals from bottom to top of the spectrum. The measured intensity gradient was found to be the same at 400, 450, 550, 600, and 650 mμ. The energy distribution with wave-length is shown in Figure 8 and the intensity gradient in Figure 9.

Figure 10 upper left shows a photograph of an experiment with *Dunaliella salina*. The phototactic reaction of *Dunaliella salina* in this experiment was negative, and instead of collecting at the effective part of the spectrum the organisms swam away from it.

Since the projected spectrum is not corrected for equal numbers of quanta at different wave-lengths, and since the intensity gradient is not linear from bottom to top, the action spectrum of phototaxis observed by the boundary of random motion is approximate only. To obtain the true action spectrum as defined at the beginning of this paper, corrections must be made by use of the curves in Figures 8 and 9, and the resulting curve calculated for numbers of incident quanta rather than incident energy. Such corrections were made in the determination of the action spectrum curves presented below.

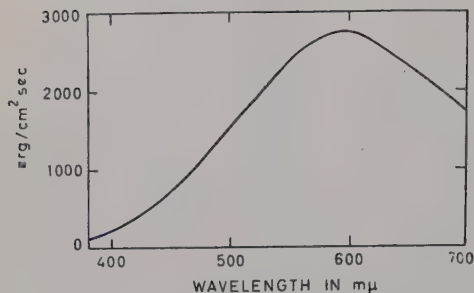


Figure 8.

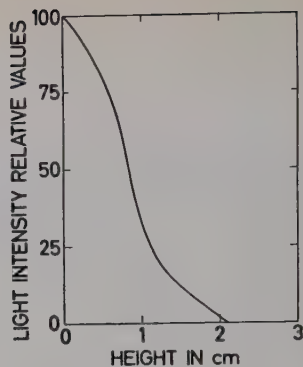


Figure 9.

Figure 8. The energy distribution with wave-length as measured 4 mm. above the bottom of the spectrum.

Figure 9. The intensity gradient from the bottom to the top of the spectrum, relative scale.

Remarks on Methods and Apparatus

With the point-by-point method it was noticed that when the organisms were balanced between the two beams, one of the beams could be varied in intensity by about 20 per cent without detectable effect on the distribution pattern of the organisms. This result was confirmed more directly when the region with random motion was observed in a set-up as illustrated in Figure 2, where the intensity gradient was calibrated in relative values, and different intensities were tested. However, the exact figure is difficult to determine due to diffuse boundaries. This insensitivity to changes in light intensity indicates that the topo-phototactic organisms are subject to Weber's law: that the smallest change in the magnitude of a stimulus which will call forth a response, always bears the same proportion to the whole stimulus. This has been shown to be true (to an approximation) for *Chlamydomonas* by Desroche (1912) and others; and for the purple bacteria by Schrammeck (1934) and Manten (1948). Thus, when working with a point-by-point method, two values have to be recorded as was pointed out by Manten for the purple bacteria. The upper and lower intensity between which the organisms will swim by random motion should be measured and the mean taken.

For the method with the projected spectrum (p. 122, Figure 3) at a certain wave-length we will have the distribution within the vessel as shown in Figure 2. At a high intensity of this wave-length the organisms will swim toward the spectrum, and at low intensity they will be attracted by the reference beam. In the region with random motion, the intensity variation will be of the order of 20 per cent. What is recorded in this method is based upon the Tyndall effect of the organisms standing against the wall illuminated by the spectrum (see Figure 3). As the organisms which actively collect at the wall greatly outnumber those which stay there by random motion, the lower limit of no reaction actually is recorded. The lower limit of no reaction will coincide with the mean region of no reaction and thus represent the action spectrum of phototaxis, as only relative values are considered for these curves.

When the action spectra of phototaxis were measured by the point-by-point method, the reference beam was kept constant in intensity and wave-length for each organism, though different reference beams sometimes were used for different organisms. A number of checks, by which both the intensity and the wave-length of the reference beam were varied, were taken in order to test whether the curve based upon measurements at one wave-length and intensity of the reference beam is representative for the entire action spectrum of phototaxis. In addition to the monochromators mentioned earlier, other light sources were used in these checks. Bands of various wave-lengths were isolated from tungsten lamps by means of glass filters and interference filters. Spectral lines at 436, 546, and 578 m μ from a Philips' HP 125 W mercury lamp were also isolated by means of Schott filters. The values of phototactic effect obtained by these measurements, proved to be independent of the wave-length and intensity of the reference beam, which means that the measurements were all taken below the saturating light intensity for phototaxis in these organisms.

A similar check was taken for the method with the projected spectrum. In this test the intensity of the reference beam and the projected spectrum were both reduced by the same value, and action spectra of negatively reacting *Dunaliella salina* recorded. The lowest intensity tested was 6 per cent of the original. No change in the different curves representing the action spectra of phototaxis was observed, which proves that even the highest intensity of the reference beam used in these experiments was below the saturating light intensity for phototaxis in *Dunaliella salina*. There is every reason to believe that this was the case in all the species examined. With decreasing light intensity, the boundaries became more and more diffuse. Because of this, the full light capacity of the apparatus was used in all measurements.

The phototactic response of negatively reacting *Dunaliella salina* to a spectrum which was somewhat altered in its spectral composition was examined in an experiment where the spectrum was screened by a film of living cells of *Dunaliella salina*. The film of living cells was obtained by illuminating a vessel containing a dense culture of negatively reacting *Dunaliella salina* uniformly from one side. This illumination fastened the cells to the opposite wall and their position was not changed during subsequent exposure. This vessel was then inserted in the beam of the projected spectrum and the collection of negatively reacting *Dunaliella salina* recorded. A film of living cells thus acted as a filter in the optical system. The results are given in Figure 10; the upper left shows the "action spectrum of phototaxis" in this organism, and the upper right shows the collection recorded when screened by the organism itself. Curve A is the boundary of the upper left photograph corrected by use of the curves of Figures 8 and 9 and adjusted for numbers of incident quanta; B is the same for the upper right. When the difference between these two curves is taken, the result should be the light which is absorbed by the screening cells; in other words, the absorption spectrum of *Dunaliella salina* at these wave-lengths. When the curve representing this difference (C) is compared with the absorption of living cells in Figure 16, the curves are in fair agreement, which shows that the set-up has good resolving power.

In addition to giving information on the sensitivity of the method, this experiment also shows how the action spectrum of phototaxis will change if the measurements are influenced by screening of the organisms themselves. This sort of distortion will result if too dense samples are used in the point-by-point method, with the vessel

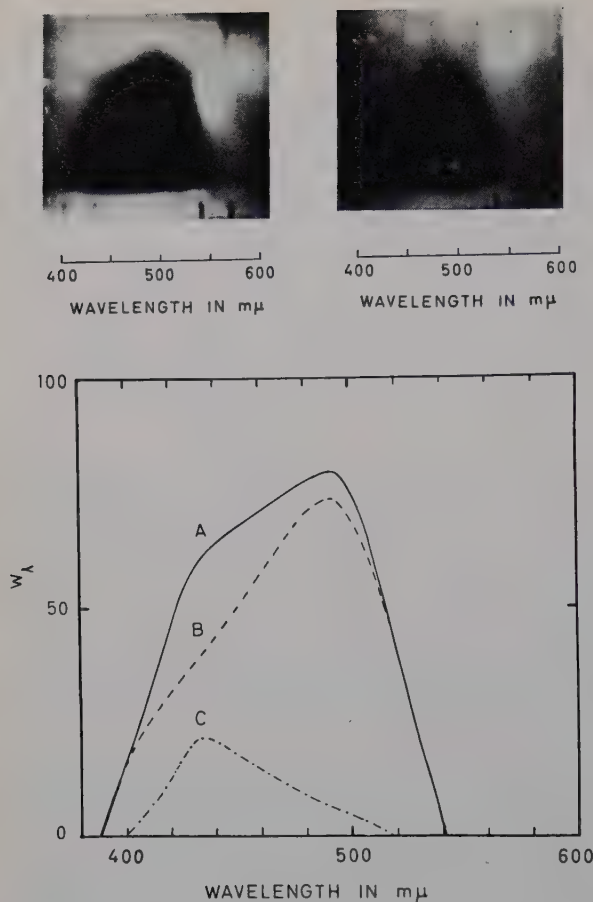


Figure 10. *The screening effect of living cells. Upper left: collection of negatively reacting Dunaliella salina in the set-up with the projected spectrum. Upper right: collection of negatively reacting Dunaliella salina when the spectrum was screened by a film of living Dunaliella salina cells. Lower part: A is upper left and B upper right, corrected according to the curves in Figures 8 and 9, and finally adjusted for quanta; C is A minus B.*

shown in Figure 4. However, relatively dilute samples were used in such measurements. In the method with the projected spectrum, distortion is avoided by the method itself, as the action spectrum here is recorded at the wall of the vessel illuminated by the spectrum and the organisms are thus allowed to be distributed according to their response at a place where the beam is not subject to any absorption by cells. An absorption similar to this also takes place naturally within the cells. This matter is discussed later (p. 147 and 148).

Materials

Volvocales

Dunaliella salina (Dunal) Teodoresco (Gibor's strain)

Dunaliella viridis Teodoresco (Gibor's strain)

Dunaliella cf. euchlora Lerche (Halldal's strain) (isolated from sea water near Hopkins Marine Station, Pacific Grove, August, 1955. Det. R. Lewin)

Platymonas subcordiformis (Wille) Hazen (Gibor's strain)

Stephanoptera gracilis (Artari) Smith (Gibor's strain)

Dunaliella, Platymonas, and Stephanoptera belong to the order Volvocales, and following the systematics of Fritsch (1948) this order is placed among the Chlorophyceae. All three are unicellular, non-colonial forms. The cells are flagellated and move actively during the vegetative phase of their life cycle. Dunaliella and Stephanoptera have two flagella at their anterior end, while Platymonas has four. They all have a stigma which is located in the anterior half.

These species were grown in "Erd-Schreiber" (Føyn 1934). The cultures were unialgal, but in our experiments sterile technique was not used.

Dunaliella salina is one of the organisms causing the red color of brine ponds. However, when kept at intermediate salinity, the species has a green color (Gibor 1955). In our experiments, only this green variety was used (see absorption spectrum, Figure 16).

It has been possible to some extent to give the cultures different treatments that will give rise to cells having either positive or negative phototaxis with a certain threshold value for phototactic response. It is important in the projected spectrum method to keep the phototactic sensitivity of the organisms under analysis above a certain value. If this is not done, complications in the measurements will arise, since very sensitive cells will react to scattered light from the vessel, particles, and other organisms in the water. This matter is discussed later in this paper (p. 138). The treatment that was followed varied somewhat from species to species. However, in general, a dense culture which was kept for one day in the vessel in which the measurements were to be carried out would contain cells with positive phototaxis and a suitable threshold value. A somewhat more dilute culture was apt to contain cells which were too sensitive. A culture suitable for measurements of the negative phototaxis was obtained by adding some fresh medium to a dense culture. If too much medium was added, the threshold value would be too low.

Ulva-gametes

Ulva rigida Agardh

Ulva taeniata (Setchell) Setchell & Gardner

(Both identified by G. J. Hollenberg)

Ulva-gametes swim actively with two anterior flagella. A chloroplast and a stigma are present in the cell.

Fertile specimens of these species were collected at sunrise near Hopkins Marine Station, Pacific Grove, in August, 1955. After collection, they were wrapped in plastic and carried to the laboratory. Fertile thalli collected in this way usually discharge gametes immediately after transfer to sea water (Smith 1947). The gametes were discharged in a jar with sea water and collected by their positive phototaxis. The experiments on their phototaxis were carried out on the day of collection.

Dinophyceae

Goniaulax catenella Whedon & Kofoid (Halldal's strain) (Isolated near Hopkins Marine Station, Pacific Grove, August, 1955).

Peridinium trochoideum (Stein) Lemmermann (Nordli's strain)

Prorocentrum micans Ehrenberg (Nordli's strain)

Since the morphology of Volvocales and Ulva-gametes is rather well known it is summarized only briefly in this paper. On the other hand, the Dinophyceae are much less known. There exists in this group a great morphological variety which is not dealt with in botanical text books. Some of the discussion in later sections of this paper is based upon the morphology of the species mentioned above. They will therefore be described in some detail.

Goniaulax and *Peridinium* are related genera which are similar in appearance. A sketch of *Peridinium trochoideum* is shown in Figure 11. This description also applies to *Goniaulax catenella*. *Peridinium trochoideum* has a cellulose envelope composed of a definite number of regularly arranged plates, and is provided with two furrows, one transverse and the other longitudinal. The two flagella emerge close together through apertures in the envelope, where the furrows meet. One of the flagella is band-shaped and encircles the individual in the transverse furrow. The other flagellum is a thread considerably longer than the cell, and is directed backwards during movement. The function of these two has been studied in detail by Metzner (1929). Both flagella take part in driving the organism forward, but in addition to this movement, a rotation around the longitudinal axis of the organism occurs also. The latter motion is caused mainly by the transverse flagellum. No stigma (or ocellus) has been observed in *Peridinium trochoideum* (nor in any of the armored Dinophyceae). It is present in some unarmored marine forms (Kofoid and Swezy 1921) and in some fresh-water species related to *Peridinium*. In these cases it is situated near the point where the furrows meet (Fritsch 1948). Metzner (1929) reported that this point is photosensitive in *Ceratium cornutum*, a fresh water species without a stigma. It is commonly believed that this is the photosensitive part of the cell within this group as a whole.

Prorocentrum micans is less specialized than *Goniaulax catenella* and *Peridinium trochoideum*. The cellulose envelope is composed of two distinct halves. The suture joining them runs longitudinally (Figure 12). The cell is strongly flattened in a plane parallel to the suture. The two flagella emerge apically through a pore which occurs as an emargination in one of the valves. One, which is thread-like and directed anteriorly, pulls the individual forward, while the second band-shaped undulatory flagellum swings transversely about it and causes a rotation of the individual. No stigma has been reported in *Prorocentrum* nor in any of its related forms. *Goniaulax catenella*, *Peridinium trochoideum*, and *Prorocentrum micans* are photoautotrophic forms. *Goniaulax catenella* and *Peridinium trochoideum* have a number of yellow-brown chromatophores at the periphery of the protoplast, *Prorocentrum micans* has two chromatophores.

These species were grown in "Erd-Schreiber" (Føyn 1934). The cultures were unialgal, but not free from bacteria.

Compared with the Volvocales, the armored Dinophyceae are difficult to grow and maintain in a condition to give good phototactic response. Like the Volvocales, the Dinophyceae will react negatively or positively, or show no phototaxis, depending upon the condition of the cells. Negatively reacting Dinophyceae are very unprecise in their swimming direction. Measurements of negatively reacting Dinophyceae have therefore been omitted. However, some checks which have been taken strongly indicate that the action spectra for positive and negative phototaxis are identical in these flagellates, as was the case for the Volvocales. The general culturing procedure

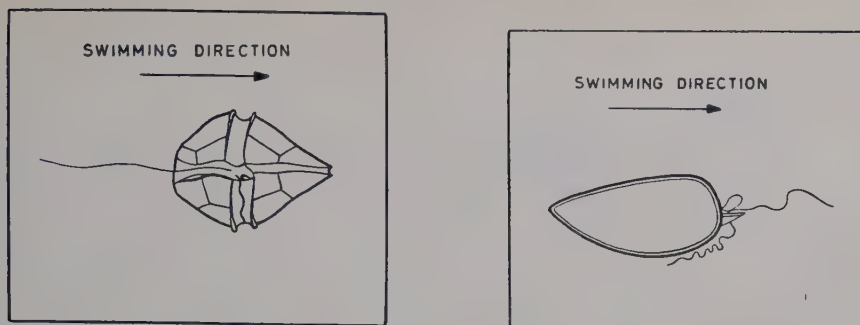


Figure 11. Sketch of *Peridinium trochoideum*. Figure 12. Sketch of *Prorocentrum micans*.

was as follows: an inoculum added to about 25 ml. of medium started in a 50 ml. Erlenmeyer flask was allowed to grow to a dense culture having a brownish yellow color. This culture was then transferred to about 250 ml. of medium and allowed to grow to a high density after which it was transferred to about one liter of medium. The samples for the measurements were taken from this flask. Fresh "Erd-Schreiber" medium was added to the flask every other day, and the culture transferred to a new flask about every other week. The cultures were illuminated from above by fluorescent tubes giving an intensity of about 1,000 lux.

Action Spectra of Phototaxis

Volvocales

The action spectra of phototaxis in the *Volvocales* were determined by the point-by-point method with the monochromator at Hopkins Marine Station, and by the projected spectrum method.

In the point-by-point measurements, the organisms were changed in the vessel about every half-hour, but several measurements were taken using the same sample. Measurements were taken every 5 m μ over the phototactically effective part of the spectrum. When the organisms were balanced between the two light beams, checks were taken on both sides of this intensity in order to establish the mean value causing the random motion stage. Owing to the fact that the exact value of the intensity causing the random motion stage is difficult to establish, some variations occurred in our measurements. The experimental error can, of course, be lessened by taking a great number of measurements. Greater precision was more easily achieved, however, by using the projected spectrum method.

By the point-by-point method, action spectra of positive phototaxis were determined for *Dunaliella viridis*, *Platymonas subcordiformis*, and *Dunali-*

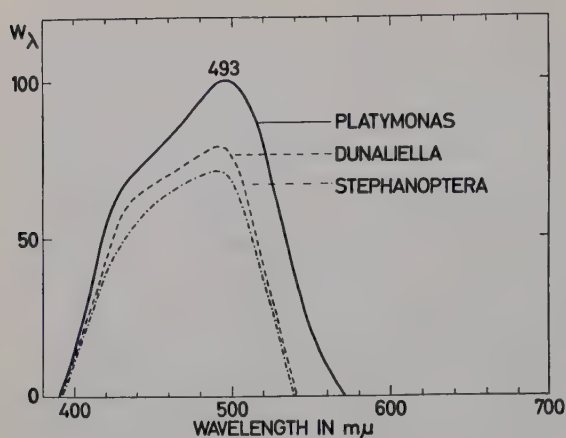


Figure 13. Action spectra of phototaxis in *Volvocales*.

ella cf. euchlora. The action spectra of negative phototaxis were determined for *Dunaliella salina* and *Stephanoptera gracilis*.

The action spectra of both positive and negative phototaxis have been measured for all the species with the projected spectrum method.

A number of experiments were performed for each species and the results recorded either by the photographic method or by manual tracing. In many cases the results obtained from several experiments with the same species were identical; in other experiments small deviations occurred. Some experiments resulted in very distorted patterns which were caused either by convection currents in the vessel or by using overly sensitive organisms. These results were rejected.

To obtain the final action spectrum of phototaxis, all the accepted curves were traced on the same paper and the mean curve was drawn. This curve was then finally corrected as mentioned on p. 125. The results are shown in Figure 13. The action spectra of phototaxis in the three *Dunaliella* species showed no significant difference and are represented by the same curve. In considering the relative values, the phototactic activity of *Platymonas* at 493 mμ is set at 100. The spectral region of phototactic activity is from about 400 to 570 mμ for *Platymonas* and from 400 to 540 mμ for the others. All of the species show a maximum at 493 ± 3 mμ and a small shoulder at around 435 mμ.

The results from the point-by-point method show the same over-all curves for *Platymonas* and *Dunaliella cf. euchlora*, while for the other two *Dunaliella* species and *Stephanoptera*, a minimum was indicated at 460 mμ. This minimum was never recorded in the projected spectrum method and is not considered significant.

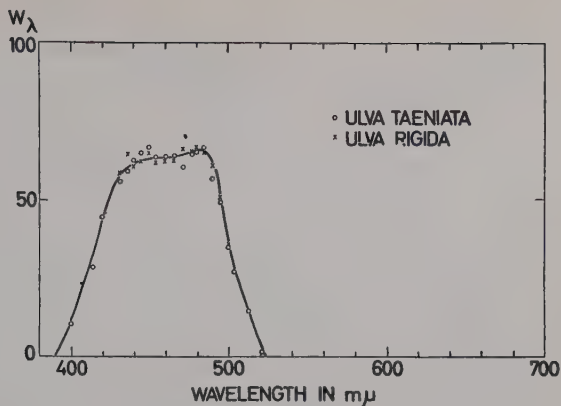


Figure 14. Action spectrum of phototaxis in *Ulva*-gametes.

Ulva-gametes

The action spectra of phototaxis in the *Ulva*-gametes were only measured by the point-by-point method, using the monochromator at Hopkins Marine Station (p. 122). The phototactic reaction of the gametes was positive. The procedure during the measurements was the same as described for the point-by-point method in the *Volvocales*. The results are given in Figure 14. There is no indication of any difference in the action spectra of phototaxis in the two species and they are represented by the same curve. In this figure, as in Figure 13, the phototactic activity is referred to that of *Platymonas* at 493 mμ as 100. Phototactic activity was recorded between 400 and 520 mμ with a maximum at 485 mμ and a shoulder at 435 mμ. There is some indication of a small minimum at around 460 mμ, but conclusions as to any detail in this region of the spectrum are not possible.

Dinophyceae

The action spectra of phototaxis in the *Dinophyceae* were determined by the point-by-point method with the monochromator at the Carnegie Institution and the method with the projected spectrum. In addition to the two methods mentioned above, glass and interference filters were used in preliminary experiments at the University of Oslo.

When the action spectrum of phototaxis of one of the species was to be measured by the point-by-point method, the reaction of the organisms was first checked for positive response at intermediate light intensity, and samples were then transferred to the vessel illustrated in Figure 4, for measurement. The readings were taken five minutes after the exposure. In order to obtain good results, it was necessary to renew the sample for each intensity tested.

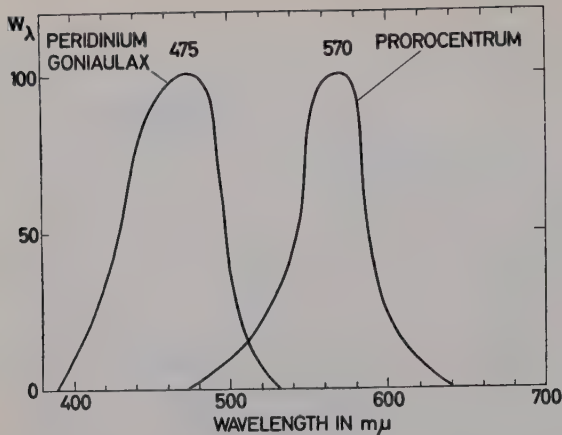


Figure 15. Action spectra of phototaxis in *Dinophyceae*.

If this had not been done, the organisms might have changed their reaction to negative or they might have become non-phototactic in ways that were difficult to predict. Measurements were taken at 5 mμ intervals over the phototactically active part of the spectrum. These organisms are less precise than the *Volvocales* and the *Ulva*-gametes in their phototactic line-up, and therefore, greater deviations occurred in the experimental results. As in the case of the *Volvocales*, this difficulty was remedied by using the projected spectrum.

Also in the method with the projected spectrum, only positive action spectra were recorded. The vessel in which the measurements were to be carried out was filled some hours in advance with a dense culture showing positive phototaxis, and then placed in the apparatus for measurement. The reading was taken after thirty to sixty minutes exposure. A number of experiments were carried out for each species.

The action spectrum of phototaxis was obtained as described for the *Volvocales* (p. 125). The results are given in Figure 15. Since no difference was recorded in the action spectra of *Goniaulax catenella* and *Peridinium trochoideum*, they are represented by the same curve. The curves are adjusted to 100 units at their maxima. *Goniaulax* and *Peridinium* both show maxima at 475 ± 7 mμ, and *Prorocentrum* at 570 ± 7 mμ. In the experiments with the point-by-point method, a minimum was indicated at 460 mμ for *Peridinium* and at 550 mμ for *Prorocentrum*. But neither of these minima were recorded by the projected spectrum method and are not considered significant. Due to the fact that the boundaries for the phototaxis are less distinct than for the *Volvocales*, little can be said about minor details in the curves. A small shoulder is indicated near 440 to 450 mμ for *Goniaulax* and *Peridinium*, but there is no indication of any irregularity in the curve for *Prorocentrum*.

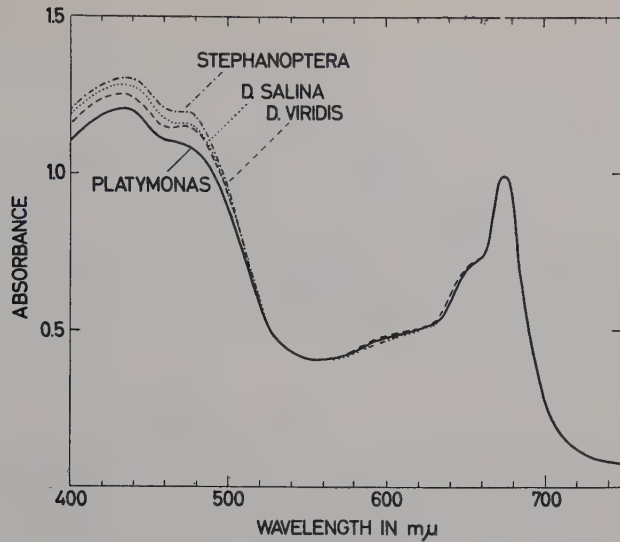


Figure 16. *Absorption spectra of living cells of Volvocales.*

Absorption Spectra of Living Organisms and Pigment Analysis

Volvocales

The absorption spectra of living cells were recorded by the method of Shibata *et al.* (1954). In order to simplify the measurements, the cells were concentrated by centrifugation at $700\times g$ for five minutes and a thin layer of cells was painted on a piece of filter paper (Schleicher and Schüll No 589) and the absorbance of this painting measured. Unpainted filter paper was used as a blank. This procedure was suggested by K. Shibata (personal communication). The same curves were obtained by this method as for suspensions of living organisms when the result of the latter was corrected for distortion caused by sedimentation of cells (see p. 136). The results are given in Figure 16. The different curves were made to coincide at 675 $m\mu$ which was the position of the red absorption peak for all these species.

The differences in the yellow-red region of the spectrum were small. They were somewhat greater in the blue. The great similarity in the shapes of these curves indicates, however, that the major pigment composition is the same in these different species. At the place where the action spectra of phototaxis in these species all have a maximum (493 $m\mu$) there is no indication of the presence of a particular pigment specifically effective in phototaxis.

The fat-soluble pigments in the Volvocales have not been analyzed, but the absorption spectra of living cells suggest that none of the major carot-

enoids has a peak around 493 m μ . It is also unreasonable to assume that any of the carotenoids known from other Chlorophyceae will have a major maximum at this wave-length *in vivo*. For a review of the carotenoids see Goodwin (1954) and Karrer and Jucker (1950).

A few attempts have been made to analyze the water-soluble pigments of the Volvocales. The water extract was pale yellow and its absorption curve had a maximum in the blue-violet part of the spectrum. The absorption spectrum, however, did not reveal any detail indicating the presence of a pigment absorbing around 493 m μ ; therefore, separation of the water-soluble pigments was not attempted.

Dinophyceae

The absorption spectra of live cells of *Peridinium trochoideum* and *Prorocentrum micans* were measured by two methods: a) the method already described that was used for the Volvocales (see p. 135), and b) the opal-glass method (Shibata *et al.* 1954) using a Cary Recording Spectrophotometer.¹ Both methods gave identical results.

By the latter method individual absorption curves were distorted by the rapid settling of the cells. The distortion was minimized, however, by averaging the curves obtained by quickly shuttling the spectrophotometer several times between longer and shorter wave-lengths. Before each run, the cells were uniformly suspended by rocking the cuvette. The results are given in Figure 17. The curves for the two species were made to coincide at 675 m μ which was the position at their red absorption peak.

A difference in the absorption spectra corresponding to the difference in their action spectra of phototaxis was not observed. Below 650 m μ , *Peridinium trochoideum* had a higher absorption than *Prorocentrum micans* due to a higher carotenoid content, but the peaks and shoulders are similar in position. Thus, their absorption spectra did not reveal any qualitative difference in the pigmentation of these two species.

The fat-soluble pigments were extracted from *Peridinium trochoideum* and *Prorocentrum micans*, and separated by means of paper chromatography with the solvents used by Bauer (1952). The cells were concentrated by centrifugation, and the extraction of the pigments was effected according to Strain *et al.* (1944) using methanol and 0.5 per cent dimethylanilin. Identical paper chromatograms were obtained for both the species. The results are given in Figure 18. In order to identify the pigments, a number of chromatograms

¹ The Cary Recording Spectrophotometer was made available at Sentralinstitutt for industriell forskning, Oslo-Blindern, Norway.

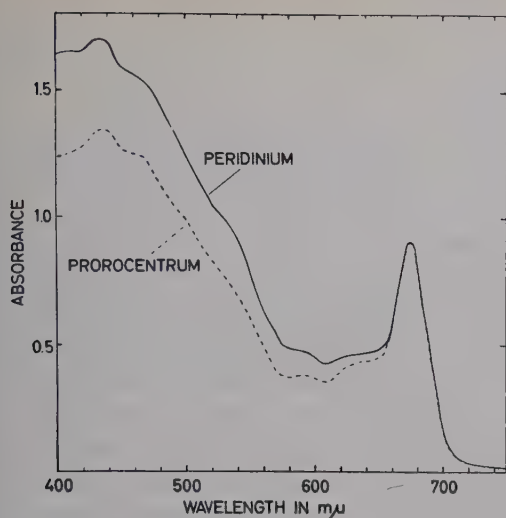


Figure 17.

Figure 17. Absorption spectra of living cells of *Dinophyceae*.

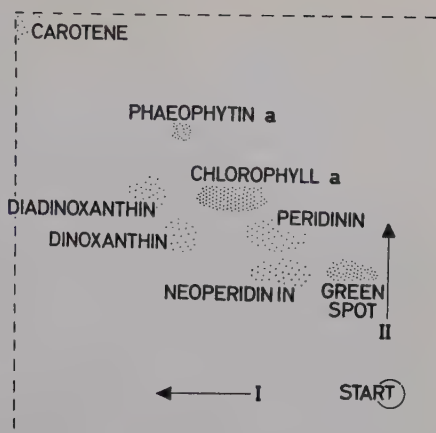


Figure 18.

Figure 18. A two-dimensional paper chromatogram of the fat-soluble pigments of *Peridinium trochoideum* and *Prorocentrum micans* developed with: I: extract benzine-petroleum ether-acetone, 10 : 2.5 : 2. II: extract benzine-petroleum ether-acetone-methanol, 10 : 2.5 : 2 : 0.25.

were produced and the pigments eluted in ethanol and their absorption spectra measured in a Cary Recording Spectrophotometer. The different pigments were thus identified by their characteristic absorption curves in this solvent (see Strain *et al.*). Although phaeophytin *a* could not be identified in the eluate from the spot so named in the chromatogram, it was identified as such from its *R_f* value (*cf.* Bauer). Chlorophyll *c*, which the *Dinophyceae* contain in small quantities, was neither revealed in the chromatogram nor in the absorption spectra of living organisms. The absorption spectrum of the pigment producing the spot marked "green spot" was similar to that of chlorophyll *a*. This pigment is probably chlorophyllide *a* according to a personal communication from J. H. C. Smith.

The water-soluble pigments in the *Dinophyceae* did not reveal any characteristic absorption at the wave-lengths where their action spectra of phototaxis had maxima. Cell samples obtained from about four-liters cultures and having a dry weight of from 0.1 to 0.5 g. have been analyzed. These samples were inadequate for satisfactory analysis. To produce adequate samples, it will be necessary to grow algae in mass cultures.

Discussion

Phobo-phototaxis, Topo-phototaxis, and Pseudo-topo-phototaxis

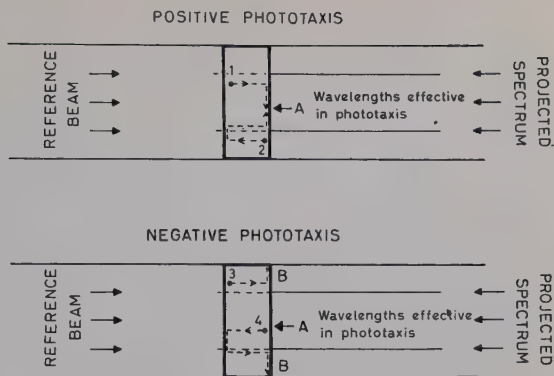
In the introduction we distinguished between phobo-phototaxis and topo-phototaxis. There is some disagreement in the explanations for the mechanism of light orientation of topo-phototactic algae. Mast (1911), Jost (1913), Jennings (1906), and others, consider only *one type of reaction, namely phobo-phototaxis*. They explain topo-phototaxis as consisting of a series of simple phobic reactions by which the organisms orient step by step in the light, resulting in a swimming to or from the light (pseudo-topo-phototaxis, Jennings 1914). On the other hand, Bancroft (1913) and Buder (1919) state that *both phobo-phototaxis and topo-phototaxis exist within the same organism; e.g. Euglena and Chlamydomonas*; they consider them to be two different types of reaction independent of each other. Buder further states that the orientation in the light beam is a result of a curved movement rather than steps of phobic reactions.

From our experiments with the projected spectrum, it was shown that movements, both parallel and normal to the light path could occur. In this method, the organisms were illuminated from one side with the reference beam which exposed them with light, uniform in phototactic effectiveness. Co-axial to this, from the opposite side, they were exposed to light which had two different gradients of phototactic effectiveness. One was for wave-length with the optimum effect where the action spectrum of phototaxis had a maximum; the other was for intensity where the optimum effect was at the bottom of the spectrum.

Assuming, as has been done on p. 121, a strictly topo-phototactic reaction, the organisms will move exclusively in the light path. They will not move normal to the light path along either of the two gradients mentioned above and will be collected in a way which allows us to determine their action spectrum of topo-phototaxis directly. Under certain conditions a distribution determined only by their reaction to light direction was obtained, which could be checked with results from the point-by-point method.

In other experiments, however, there have been aggregations at the bottom of the spectrum for the positively reacting *Volvocales*, at wave-lengths where the action spectrum of phototaxis has a maximum. For negatively reacting *Volvocales* there have been, in some experiments, collections near the walls of the vessel, parallel to the light direction. In these cases, response to the light direction was observed first. After the organisms were collected at the walls illuminated by the reference beam and the spectrum, movements normal to the light direction followed. On several occasions, the movements

Figure 19. The movements of very photo-sensitive cells when using the projected spectrum method. A is the most effective part of the spectrum and the collection point in positive phototaxis; B is where the cells collect in negative phototaxis. For further explanation see text.



normal to the light direction were so pronounced that the pattern of topophototaxis was completely distorted and could not possibly be recorded. In other experiments it was scarcely noticeable, evidently depending on the condition of the cells.

On all occasions where movement normal to the light direction occurred, the cells were extremely sensitive to the light. Though no attempts have been made to determine the threshold value under different conditions, it is no doubt highly variable. Variations in this factor can explain the different movements in the following way: when light from the reference beam and the spectrum entered the vessel, secondary light sources arose, due to scattered light from the vessel and from particles and organisms in the water. When the threshold value for phototactic response was low, the algae, in addition to their main response to direct light, also reacted to scattered light. Cells having such a low threshold value, when exposed to the light combination of the method with the projected spectrum, moved as illustrated in Figure 19. Only horizontal movements are shown in this figure.

In Figure 19 (1) is a cell showing *positive* phototaxis being within the part of the spectrum which is more effective in phototaxis than the reference beam. This cell will be attracted by the light from the projected spectrum and swim to the wall illuminated by the spectrum. When the swimming in the light direction is hindered, its movement will be directed by scattered light and it will swim toward the most effective part of the spectrum, A, and stay there. If (2) is a *positively* reacting cell within the part of the spectrum where the reference beam is most effective, it will swim toward the wall illuminated by the reference beam. When further swimming toward the light is hindered, it will be attracted by scattered light from the effective part of the spectrum and move within this region and finally follow the pathway described for (1).

When the reaction is *negative*, a cell (3) outside the part of the spectrum which is more effective than the reference beam, will first swim toward the wall illuminated by the spectrum. When further swimming in that direction is hindered, its movement will then be directed by scattered light from the effective part of the spectrum and the cell will move toward B. Finally, a *negatively* reacting cell (4), within the part of the spectrum which is more effective than the reference beam, will first swim toward the wall illuminated by the reference beam, then it will be directed out of the effective part of the spectrum and the pathway outlined for (3) will be followed.

We feel that the movements normal to the light direction, which in some cases distorted our measurements, are most reasonably explained as reactions to secondary light sources from scattered light, as outlined above (Figure 19), rather than as movements along gradients of effectiveness.

The same explanation can be applied to the experiments of Engelmann on the phototactic movement of *Euglena* in a spectrum under the microscope (Engelmann 1882 a). In this experiment, *Euglena* is only allowed to move normal to the direction of the light. The result is an aggregation in the blue-green part of the spectrum. This experiment is often taken as proof of phobo-phototactic response in *Euglena*, but using the arguments above, it might well be explained as topo-phototactic reaction to scattered light within the preparation.

The answer as to how the mechanism of light orientation in topo-phototactic algae acts, can not be considered as known. Many factors such as the site and function of the photoreceptive organ involved in this reaction; the movement of the cells and the flagella, need more analysis along descriptive lines. As long as the metabolism of topo-phototaxis is practically unknown, little can be assumed about what causes or does not cause changes in the flagellar apparatus.

We agree with Buder (1919) that the term topo-phototaxis should be used for active orientation according to light direction and a swimming determined by this direction. In which way this orientation is obtained does not concern the term, though it is important in the explanation of the whole process.

The Stigma and the Flagellar Apparatus

The flagellar apparatus of Volvocales and Dinophyceae has been analyzed by Entz (1918) for *Polytoma*; McKater (1929) for *Chlamydomonas*; and by Jollos (1910) for *Ceratium* and *Gymnodinium*. Their results, which are briefly summarized below, are in all-over agreement with each other and in general agreement with results from investigations of the flagellar apparatus in other groups of algae (for review see Frisch 1948). This suggests that a great

similarity exists in the flagellar apparatus of all flagellated organisms. This assumption has been confirmed lately by Manton (1954), who in recent work with the electron microscope, showed that the internal structure of flagella is very similar throughout the plant and animal kingdoms.

Entz showed that each flagellum arises from a round body (basal granule) situated near the surface of the protoplast. The two granules are united by a delicate thread and may be connected to the nucleus in different ways. On some occasions no connection can be seen; on others, a thread (rhizoplast) extends from one of the basal granules to the surface of the nucleus. In simpler cases the flagella extend more directly into the cell and continue as a thread to a centrosome, or connect with the nucleus. This led Fritsch to assume that the basal granule must be considered equivalent to the centrosome; a conclusion implying a strong cytological connection between the flagella and the nucleus.

Thus, a great many investigators have been able to analyze the flagellar apparatus in detail and to prove that a connection exists between the flagella and the nucleus. *No cytological proof has been given so far that the stigma is a part of the flagellar apparatus.* However, the assumption that the stigma is a photoreceptive organ associated with the orientation of topo-phototactic algae seems to be generally accepted. Some of the evidence for such a function and the theories concerning it will be reviewed here.

The stigma usually occurs in flagellated forms but not in all of them. Phototactic reactions occur in several green forms which lack a stigma; and a number of Dinophyceae with no apparent stigma or ocellus show phototactic response also (Metzner 1929, Nordli 1957, and this paper). The coccolithophorids do not possess a stigma, but phototaxis is reported to occur within this group in *Coccolithus huxleyi* (Mjaaland 1956). Finally, phototactic response has been demonstrated in a number of colorless forms (Strasburger 1878, Luntz 1931).

As early as 1882, Engelmann (1882 a), in studies of the phototactic behavior of *Euglena* by passing a shadow over the cell, showed that the photosensitive spot was not in the colored body known as the stigma but in the colorless portion around the flagellar base. It was suggested by Wager (1900) and by Haye (1930) that the thickening on the flagellum acts as photoreceptor. Essentially, Mast (1927) agreed with this point of view but considered the stigma in *Euglena* to be divided into two portions: one colorless photosensitive body and the other containing the visible pigment. This point of view, however, does not seem to be generally accepted (for the morphology of *Euglena* see Hollande 1952, and Fritsch 1948).

Mast's explanation of the function of this organ in phototaxis is that when *Euglena* swims, it rotates around its longitudinal axis. In this way, periodic

shading of the photosensitive portion by the pigmented body, changes the rate of absorption of light by the photosensitive substance which then controls the mechanism that directs the movement. This scheme gives a somewhat reasonable explanation for the function of the stigma in *Euglena*. However, the stigma in other organisms is sometimes built in quite a different way and the explanation of its function is based upon entirely different principles.

Mast claimed that the stigma in *Volvox* and *Gonium* consists of a cup-shaped pigmented body (pigmentosa) and a lens located at the mouth of the cup. He postulated the presence of a photosensitive substance between these two portions. According to Mast the lens focuses two spots: one yellowish, within the pigmented body, and another blue, within the photosensitive substance. The pigmentosa transmits light of longer wave-lengths but reflects the blue-green from its inner surface. The latter light comes to a focus once more within the photosensitive substance and is finally projected out of the stigma. Mast claimed that it was possible to follow for quite a distance a blue-green beam excited from the stigma. A periodic shading mechanism caused by the pigmented cup, by analogy with the one described for *Euglena*, is also assumed to take place in the stigma of *Volvox* and *Gonium* when the colonies turn during the swimming.

In *Chlamydomonas* and related forms, where the stigma is situated at the surface of the cell, nearly midway between the anterior and posterior ends, still another explanation of its function was needed. The hyaline portion which contains the postulated photosensitive substance is directed outwards and lies within the pigmented cup. The hyaline portion here is not believed to act as a lens, but a similar periodic shading of the photosensitive substance by the pigmented cup should take place in the same way as described for *Euglena* and colonial forms.

There are some objections to Mast's theories of the function of the stigma in the light orienting mechanisms of flagellated forms. In *Volvox* and *Gonium* we believe he used physical laws in places where they do not apply. The different portions of a stigma are somewhat larger than the wave-length of the light, but they are of the same order of magnitude. In this case, scattering and diffraction of light will distort any sort of precise refraction or reflection by any of these parts, and chromatic aberrations as assumed by Mast can not take place.¹ However, it is possible to give a reasonable explanation for the beam of blue-green light which is claimed by Mast to be projected from the stigma when it is illuminated from a certain angle. When a body has an absorption at a certain wave-length, it will, when illuminated

¹ Personal communication from P. Latimer.

with "white" light, scatter light of longer wave-lengths (Latimer and Rabinowitch 1956). The pigmentosa usually has a yellow-red color, due to an absorption of blue light. When illuminated, therefore, it is expected to scatter blue-green light.¹ Luntz (1931) was not able to confirm Mast's observations and rejected his hypothesis concerning the function of the stigma. Luntz assumed that the light absorbing portion of the stigma is the portion with the visible pigment.

If the morphology of motile algae which belong to different systematic units is compared, it is evident that what is called a stigma in different organisms must be a number of different organs situated at different places in the cell. In *Euglena* it is situated in the colorless, anterior part of the cell adjacent to the flagellum; in *Volvox* and *Gonium* on the anterior half of the zooids, but at some distance from the flagella; in some *Chlamydomonas* and related forms, more or less at the middle of the cell; and in some *Ulva*-gametes in the posterior half (Smith 1947). The stigma may be situated at the superficial layer of the cytoplasm, but it is also found in the inner part of the cell, where it might be a part of the chromatophore or apposed to the edge of it (to one of them, if more than one is present).

If all these types of organs should have the same function in a direct way, in the mechanism that orients topo-phototactic algae according to the direction of the light, a number of explanations based on different principles must be applied. No attempt has been made to give a unified explanation for the action of the stigma, and it appears unlikely that it is possible.

Our information about the stigma can be summarized as follows: *the common presence of the stigma in phototactic algae and its rare occurrence in non-motile forms, strongly indicate that this organ is involved in phototaxis. However, since a great number of forms that react phototactically evidently lack a stigma, it may not be demanded for this reaction. Whenever the relative sensitivity of parts of organisms has been tested, it proved to be highest near the flagellar base. In Euglena, the stigma lies within this region, but the light-absorbing organ has been shown not to be the colored portion, and it is doubtful whether the photoreceptor and the stigma are two portions of the photoreceptive organ. When the stigma is far away from the flagellar base there is no evidence that it is the photoreceptor or that the photoreceptor is adjacent to it. There is no cytological evidence that the stigma is part of the flagellar apparatus.*

Based upon this we suggest that the photosensitive spot in topo-phototactic algae is situated at or near the flagellar base, but is not the stigma or any organ connected to it. In what way this photoreceptor acts on the directive

¹ Personal communication from P. Latimer.

mechanisms for light is not clear, but the result is evident. With only one light source, the cell will in one case orient so that the photosensitive spot is directly illuminated; adjustments in the direction of swimming occur when a shadow falls on the spot. When the direction of response is reversed, the cell orients so that the photosensitive spot is shaded, and in this case, adjustments for the direction of swimming occur when the spot receives direct illumination. What will cause a negative or a positive reaction should consequently be determined by the morphology of the cell, provided the same mechanism occurs elsewhere.

In the Volvocales, the flagella are pointed forward during movement and the photosensitive spot is presumably at the anterior half. This is also the case for *Prorocentrum* (p. 130). In this case an orientation that illuminates the photosensitive spot leads to a positive reaction.

On the other hand, *Peridinium* and related Dinophyceae have one of the flagella directed backward during movement and the other encircles the cell in the transverse furrow. The photosensitive spot is presumably at the place where the furrows meet (p. 130). A swimming toward the light means a shading of the photosensitive spot by the anterior half. Differences in the manner of reaction between the Volvocales and *Prorocentrum* on the one hand, and *Peridinium* on the other, should therefore be expected. Comparative experiments with these two groups, directed at analyzing this difference are in progress and some results support the assumption. This work will be published later.

According to the above assumption, some sort of shading mechanism is needed both in positive and in negative phototaxis, regardless of the morphology of the cell. In other words we have to deal with two absorbing items in the mechanisms of topo-phototaxis: a) *a photoreceptor* and b) *a shading portion which when inserted in the light path has the ability to reduce the light falling on the photoreceptor*.

Let us assume that a cell with no appreciable absorption in the visible light, that is, a colorless flagellate, has a flagellar apparatus including a photoreceptor absorbing mainly in the visible region of the spectrum, but also absorbing somewhat in the ultra-violet (Figure 20). This means that the photoreceptor absorbs an equal amount of visible light, no matter how the cell is oriented according to the light source. Since the cell contains appreciable amounts of protein, and other ultra-violet absorbing components, the relative amount of ultra-violet light that reaches the photoreceptor will depend upon the orientation of the cell.

There are reasons to believe that Weber's law is valid in phototaxis (see p. 126). Therefore, in order to induce a response, we assume that the intensity of the light effective in phototaxis must be varied by at least 10 per cent.

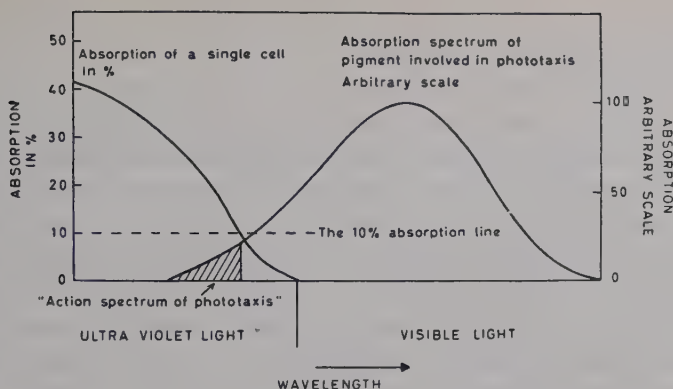


Figure 20. Schematic representation of how the action spectrum of phototaxis may be distorted in colorless flagellates assuming that the light directing mechanism in topophototaxis consists of two parts: a) a light absorber located near the flagellar base (pigment involved in phototaxis) and b) a shading body (single cell in this figure). The "action spectrum of phototaxis" will then be only within the overlapping of these two absorption spectra where the cell absorbs 10 per cent or more of the light.

If the absorption spectra of the organism and the pigment involved in phototaxis overlap (as in Figure 20), the cell will react only to light at wave-lengths in the region of this overlapping, where one single cell itself absorbs 10 per cent or more of the exciting light.

In this respect, experiments with the colorless flagellate *Chilomonas* are of interest (Luntz 1931). This flagellate is reported to have optimum phototactic response at 366 m μ , but as we have seen, this does not necessarily mean that the pigment involved in phototaxis has an absorption maximum at this wave-length, though this of course, may be true.

Of interest also are reports that *Euglena*, with no chromatophores but with stigma, are phototactic, but that those without both chromatophores and stigma do not respond to light (Hollande 1952). This, however, does not necessarily prove more than that the function of the stigma in phototaxis is other than a shading mechanism, and that its function might possibly be substituted by any other organ, say a chromatophore, which could effectively shade the photoreceptor. It is further reported that when a *Euglena*, which has no chromatophores loses its stigma, the photoreceptor disappears also (Hollande). *Euglena* is known to have an action spectrum of phototaxis with a maximum in blue-green light (Engelmann 1882 a, Mast 1917, Büning and Schneiderhöhn 1956). A colorless *Euglena* does not have a shading body in this spectral region which can activate the light directing mechanism. This might well be the explanation for the "disappearance" of the photo-

receptor in *Euglena*, which as a part of the flagellum is presumably similar in structure to a muscle.

The theory outlined above accounts for the reaction of Chilomonas in ultra-violet light; for the phototactic response of chlorophyll-free Euglena with a stigma; and for colorless Euglena not being phototactic. The colorless forms might react to ultra-violet light, but judging from the literature, this has not yet been examined. This theory also explains why a number of organisms lacking stigma, react phototactically. We do not claim, however, that the stigma in chlorophyll-containing organisms is of no importance. We believe that the photoreceptor can be effectively shaded by the chromatophores, and that the stigma is not needed in phototaxis, but in many cases it may act to improve the reaction to a high degree, especially in making the direction of movement more precise. This occurs, for example, in Euglena where the stigma is adjacent to the photoreceptor in the chromatophore-free portion of the anterior half. The stigma is located somewhat eccentric to the longitudinal axis and the photoreceptor closer to or on this axis. When Euglena swims, it rotates around its longitudinal axis. Thus, if in a positive reaction it swims somewhat at an angle to the light direction, the photoreceptor in some positions will be shaded by the stigma (for the morphology of Euglena see Fritsch 1948, or Hollande 1952) and the flagellar apparatus can, in this case, receive the necessary signal for an adjustment of the swimming direction. Due to the position of the stigma in relation to the photoreceptor, the deviation of the movement from the light direction will be very small; without a stigma it must be expected to be much greater. The stigma in other organisms, where it is close to the flagellar base, may function in the same way. More problematic is the function of a stigma situated far away from the flagellar base. However, as the stigma, due to its high content of carotenoids, absorbs blue-green light more effectively than does a "pure" green chromatophore. it might be more effective in the shading of the photoreceptor which, judging from action spectra of phototaxis in most cases has a photosensitive pigment absorbing in the blue-green. Thus, even when the stigma is located far away from the flagellar base, it might improve the phototactic reaction by its more effective shading.

It was mentioned earlier that there was no cytological evidence that the stigma is a part of the flagellar apparatus, but as we have seen, participation by the stigma in phototaxis can be assumed without demanding such a connection.

Analysis of the Action Spectra of Phototaxis

In the preceding chapter an outline was given for the orientation mechanism in topo-phototaxis which was based upon a photoreceptor and a screening

body with the ability to reduce the illumination of the photoreceptor by at least 10 per cent. If the cell, or any part of the cell, does not have this ability at all wave-lengths where the photosensitive pigment absorbs, identity between the action spectrum of phototaxis and the absorption spectrum of the pigment (or pigment complex) can not be expected. A single cell with normal chlorophyll content is expected to absorb at least 50 per cent of the light incident upon it, at wave-lengths around 675 m μ (Duysens 1952). Judging from the absorption spectra of living Volvocales and the Dinophyceae cells, the absorption of one single cell should be 20 per cent or more at any wave-length shorter than 690 m μ . The absorption for the *Ulva*-gametes is also expected to be safely above the 10 per cent level at all these wave-lengths. *We assume, therefore, that the action spectra determined do not have distortions due to insufficient shading of the photoreceptor.*

The site of the photoreceptor is assumed to be near the flagellar base, but whether it is located near the surface of the cell or farther inside it, is uncertain. The chloroplast in the Volvocales is cup-shaped, and when the anterior end is illuminated co-axially to the longitudinal axis of the cell, the chloroplast will not shade the photoreceptor. The stigma is somewhat apart from the flagellar base, and therefore we assume that, at least in one position, the photoreceptor will receive light that has not been screened by any of the major pigments present in the cell. *Dunaliella salina* (Gibor's strain), when kept at intermediate salinity, has a green color similar to *Dunaliella viridis*, *Stephanoptera gracilis*, and *Platymonas subcordiformis* (Figure 16). But even red *Dunaliella salina* does seem to differ from the green specimens in its wave-length response for phototaxis (Blum and Fox 1932).

It was also shown that the action spectra of positive and negative phototaxis were identical in the same species. This, therefore, leads us to assume that the Volvocales in their negative reaction tend to orient so that the photoreceptor is shaded from direct illumination. If the light, which is partly absorbed by passing through the cell, should be effective in negative phototaxis, a difference between positive and negative action spectra of phototaxis should result. This should be similar to the difference between the true action spectrum and that which results when the cells are screened by other cells (see Figure 10). However, such a difference has not been indicated. *We therefore assume that in the Volvocales and the Ulva-gametes the action spectra presented are not distorted to any noticeable degree by the screening of pigments within the cells.*

In the method with the projected spectrum, the mutual screening of organisms is avoided by the method itself, and as the samples used in the point-by-point method were relatively dilute, the mutual shading effect here is probably very small, if any. We therefore conclude that *the action spectra*

of phototaxis in the *Volvocales* correspond to the absorption spectra of the photoreceptive pigment (or pigment complex) in phototaxis.

As the action spectra of phototaxis in the *Ulva*-gametes have only been measured with the point-by-point method, the accuracy is less than that for the *Volvocales*. The difference between action spectra of the *Ulva*-gametes and the *Volvocales* is small, and perhaps due to experimental error. All these action spectra have more or less the same shape, with a shoulder around 435 and a maximum around 490 m μ . This suggests, therefore, that the same pigment is involved in the phototaxis of all these forms.

Due to the shape and the site of the chromatophore in the *Volvocales* and *Ulva*-gametes, we assumed that the photoreceptor, at least in one case, is not shaded by the chromatophore. This, however, is not necessarily the case for the *Dinophyceae*. In *Goniaulax catenella* and *Peridinium trochoideum* there are a number of chromatophores at the periphery of the protoplast. In both these species and in *Prorocentrum*, which has two chromatophores, the fat, which these forms store as reserve food, is yellow-brownish in color. In the *Dinophyceae* the cytoplasm is often colored violet, blue or red. The nature of these pigments is not known. If, therefore, the photoreceptor is somewhat in the inner part of the cell, there is a possibility that a screening effect due to shading of pigments within the cell may occur. As the relative content of the different pigments may vary from one part of the cell to the other, it is not possible to determine from the absorption spectra of living organisms what influence this shading effect may have on the measurements of action spectra of phototaxis.

Peridinium and *Goniaulax* have a phototactic response in the blue region of the spectrum. We were not able to distinguish between their action spectra (Figure 15). The *Dinophyceae* are much less distinct in their phototaxis than are the *Volvocales* and the *Ulva*-gametes. The accuracy of the measurements is on the order of ± 7 m μ for wave-length. However, it seemed almost certain that the drop-off in the blue-green region came at a shorter wave-length than it did for the *Volvocales*. This effect, however, might have been caused by screening pigments within the cell. Therefore, we cannot state positively that the pigment involved in the phototaxis in *Peridinium* and *Goniaulax* is different from that involved in the phototaxis of the *Volvocales* and *Ulva*-gametes.

Prorocentrum micans is unique in its phototactic response, having an action spectrum with a maximum in yellow light at 570 m μ . All other measurements of phototaxis in algae have revealed a response to blue-green or ultra-violet light. The pigment involved in phototaxis in *Prorocentrum* is certainly different from the pigment (or pigments) responsible for the phototaxis in the blue-green or ultra-violet light. Our experiments thus suggest that at

least two, possibly three, pigments are involved in topo-phototaxis of the algae examined by us.

The spectral sensitivity of phototactic response has been measured with different methods for a number of green forms. These measurements suggest that all the Volvocales, the Ulva-gametes, and the Euglenaceae have action spectra of phototaxis with a maximum around 485—500 m μ (Engelmann 1882 a for Euglena, Mast 1917 for Euglena and a number of Volvocales, Laurence and Hooker 1920 for Volvox, Luntz 1931 for green flagellates, and Bünning and Schneiderhöhn 1956 for positively reacting Euglena). These observations strongly suggest that the same pigment is involved in phototaxis of the green forms summarized above and those examined by us.

Bünning and Schneiderhöhn report a difference in the action spectra of positive and negative phototaxis in Euglena. However, there are some objections to their method. In their experiments the organisms were allowed to orient only normal to the direction of the incident light; thus, topo-phototactic response was only recorded indirectly (see p. 138 in this paper). The action spectrum of positive phototaxis was based upon measurements of the lowest value which caused positive response. For the action spectrum of negative phototaxis, their measurements were based upon intensities causing reversal from positive to negative phototaxis. This change has been shown to be influenced by several different factors, presumably those which affect the adenosine triphosphatase activity within the flagellar apparatus (Hall-dal 1956, 1957). It is certain that photosynthesis will affect this change, directly or indirectly. The action spectrum of negative phototaxis presented by Bünning and Schneiderhöhn has a rather broad maximum in the blue-violet region of the spectrum. If a smooth curve is drawn, instead of the more detailed one, it has a great resemblance to the action spectrum of photosynthesis of green algae in this part of the spectrum. We suspect that the measurements of Bünning and Schneiderhöhn for the action spectrum of negative phototaxis might have been distorted by the photosynthesis of the cells. At any rate their results on Euglena contradict the results of Mast (1917) and they are not in agreement with our own results for action spectra of positive and negative phototaxis in other algae.

The different attempts to identify the pigments involved in phototaxis have not been successful. We can safely conclude that none of the major pigments present in the algae is the photoreceptor in this reaction. Our only information about these pigments is their absorption characteristics in vivo, deduced from action spectra of phototaxis. We do not even know the nature of these pigments, and we will not speculate along this line.

Recently it has been possible to control the reaction of positive and negative phototaxis in the Volvocales by simple factors such as the absolute and

relative amount of Mg^{2+} and Ca^{2+} in the medium (Halldal 1957). This gives us hope for a more thorough analysis of the biochemistry of this reaction, and possibly, for an indirect approach to the question of the pigments involved.

Summary

1. Action spectra of topo-phototaxis in algae have been obtained by two methods: a) The algae were balanced for movements between two opposing beams, one reference beam which was kept constant in wave-length and intensity, and one exciting beam which was altered in wave-length and adjusted in intensity until the cells swam toward neither light source. The phototactic effectiveness of different wave-lengths was thus obtained. b) A spectrum having an intensity gradient was projected on one side of a vessel containing the algae, the other side was illuminated with light uniform in wave-length and intensity. Under these conditions the algae will collect on the side of the vessel where the spectrum is more effective than the uniform reference beam (positive phototaxis), and the action spectrum was obtained by measuring the energy and wave-length along the boundary of collection.

2. The precision of the measurements was analyzed.

3. Action spectra of phototaxis have been measured for five species of Volvocales; for the gametes of two species of Ulva; and for three Dinophyceae.

4. The Volvocales all have action spectra of phototaxis with a maximum at 493 m μ and with a small shoulder around 435 m μ . The action spectra of both species of Ulva-gametes have a maximum at 485 m μ and a shoulder around 435 m μ . Two of the Dinophyceae, *Goniaulax catenella* and *Peridinium trochoideum*, have action spectra with a maximum at 475 m μ ; the third, *Prorocentrum micans*, at 570 m μ .

5. Absorption spectra of living Volvocales and the Dinophyceae were measured. These spectra did not reveal any features indicating the presence of pigments corresponding to the action spectra of phototaxis.

6. Pigment analysis of *Peridinium trochoideum* and *Prorocentrum micans* showed that the same major fat-soluble pigments were present in both these species.

7. The action spectra of positive and negative phototaxis were identical for the same species in the Volvocales. Some observations strongly indicate that this was true for the Dinophyceae also.

8. Some of the theories about the function of the stigma in phototaxis are discussed. It is concluded that the directing mechanism in phototaxis

consists of two parts, one of which is a light absorber located near the flagellar base. In order to act in phototaxis, a shading organ is required with the ability to reduce by at least 10 per cent the light in the spectral region where the light sensitive pigment absorbs. The stigma in colored forms, when present, is assumed to act only as an auxiliary body which on some occasions will improve the precision of the movement.

9. The different action spectra have been analyzed and when the experimental errors and the screening effect of pigments within the cells are taken into consideration, it is concluded that at least two, possibly three, different pigments are involved in the phototaxis of the algae examined by us: a) the same pigment is assumed to be involved in the Volvocales and the Ulva-gametes; b) possibly this same pigment is also involved in the phototaxis of *Goniaulax catenella* and *Peridinium trochoideum*; c) definitely different from these is the pigment involved in the phototaxis of *Prorocentrum micans*.

10. None of these pigments has been isolated and even their nature is unknown.

Laboratory experiments on the phototactic behavior in Dinophyceae were started by cand. real. Grethe Rytter Hasle and myself at the Institute for Marine Biology, B, University of Oslo, Norway. Mrs. Hasle has been working since 1952 on other problems and the work on phototaxis has been continued by myself. I wish to express my sincere thanks to Mrs. Hasle for her cooperation in the first year.

In May, 1952, Mrs. Hasle and I worked on topo-phototaxis in the Biophysical Research Group of the Physical Institute of the State University, Utrecht, Holland. Many thanks are due to Dr. J. B. Thomas and his co-workers for their kind advice on biophysical methods and measurements.

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Preliminary Observations on Anthocyanins and Other Flavonoid Compounds and Respiration Rates in Different Ecotypes of *Solidago virgaurea*

By

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The biochemical and physiological background of the differentiation of ecotypes in higher plants has been studied for some time at the Institute of Genetics, Uppsala. The plant material consists of populations of *Solidago virgaurea* collected in northern and middle Europe in biotopes ranging from sea-shore to mountains above upper timberline. The plants have been growing for several years in the botanical garden of the institute and therefore the observed differences can be looked upon as mainly hereditary.

Analyses of the flavonoid compounds have shown that these substances are found in different concentrations in the separate populations during autumn — September to November. Measurements of the contents of anthocyanins and anthoxanthins in fresh leaves have been carried out using paper chromatography and absorption spectrometry.

In *Solidago* the anthocyanin consists principally of cyanidin-3-gentiobioside, but another cyanidinglycoside has also been found, though sparingly. Table 1 shows the mean values of the extinction at 514 m μ — absorption maximum of the glycoside in the visible — in extracts of representative leaf samples. In each population 4 to 5 plants were examined. "Finse" represents an alpine ecotype from Hardangervidda, Norway, and "Åreskutan" is a sub-alpine ecotype from Jämtland, Sweden. "Hallands Väderö" represents a coastal population from the southern part of the Swedish west coast and "Kivik" a coastal type from the east coast of Skåne in the southernmost part of Sweden (Turesson 1925, p. 204 ff.). Paper chromatographic analysis

Table 1. Oxygen consumption of leaf discs and relative light absorption of anthocyanin extracts of some ecotypes of *Solidago virgaurea*.

Ecotype	Oxygen consumption of leaf discs, microliters O ₂ /mg dry weight and hr.	Relative extinction at 514 mμ
"Finse", 1,350 m s.m.	3.99 ± 0.11	0.483 ± 0.029
"Myrdal", 870 m s.m.	3.90 ± 0.17	—
"Åreskutan", 900 m s.m.	—	0.340 ± 0.009
"Hallands Väderö", near sea-level ...	2.69 ± 0.12	0.073 ± 0.008
"Kivik", near sea-level.....	2.38 ± 0.04	0.080 ± 0.006

has shown that the extracts from the two latter populations lacked measurable quantities of anthocyanin. These samples have no absorption maximum at 514 mμ, and the recorded absorption can be attributed to tannins, which are also present in the extracts owing to the extraction methods.

Thus, the differences in anthocyanin contents between mountain and low-land populations of *Solidago* are evident, and these differences are the same for ten populations, which have been examined.

The same variation has been observed in the concentrations of anthoxanthins; *i.e.* in leaves from alpine and subalpine populations the concentration was higher than in populations from lower levels. A quercetin-glycoside proved to be the most important anthoxanthin.

Some saturated, aromatic acids frequently occurring in the leaves of *Solidago* are also being investigated. Some of these acids can probably be intermediate substances in the biosynthesis of flavonoid compounds. The aromatic acids that have been particularly studied are *p*-coumaric acid and chlorogenic acid, the depsid of caffeic acid and quinic acid. On the whole, the relation between the quantity of flavonoid compounds and of chlorogenic acid is inverse in the examined populations. Thus, the alpine and subalpine material has considerably lower concentration of this acid than material from other biotopes. The above-mentioned inverse relation could be explained assuming that the two groups of compounds have precursors in common.

Thus, the studied populations of *Solidago virgaurea* can be divided into two groups with regard to the observed differences in flavonoid pigments and related aromatic acids. One group comprises alpine and subalpine ecotypes, the other populations from localities at lower levels. In the first group a subalpine population from Switzerland is also included. The conformities between alpine and subalpine populations from separate areas as to the above-mentioned pigment relations indicate that these conditions may have a selective influence on the differentiation of ecotypes with heritable adaptation to alpine and subalpine environments. The more intense pigmentation

of these ecotypes may exert a positive effect by increasing the absorption of radiated heat (*cf.* Krog 1955). Little is yet known about the physiological role of the flavonoid compounds, but Szent-Györgyi's observations (Blagowestschenski 1955, p. 88) that flavonoids are necessary in some cases in respiration indicate that these substances may be of more importance than has generally been supposed.

In connection with the chemical investigations, measurements of leaf respiration were carried out using manometric methods (Eberhardt 1954, p. 255 ff.). Differences between ecotypes were observed (Table 1). "Myrdal" is a subalpine population from Hardangervidda, Norway, collected only 35 km. from the above-mentioned "Finse" population. The measurements were made separately on three normal rosette leaves from each plant. Three plants were examined from each population. In the mountain populations the leaf respiration is considerably stronger calculated both per unit area and per mg dry weight than in lowland populations. The correlation between pigment concentration and respiration rate is obvious (*cf.* Eberhardt 1954).

These differences in respiration were observed in the autumn at the end of the vegetative period, but the conditions are similar also in spring. The differences in leaf respiration disappear, however, with increasing night temperature and in the middle of summer they are quite lost. Thus, they are probably related to differences in frost hardiness and changes in sugar level connected with it.

The physiological adaptation to the temperature conditions of the biotope must be of great significance for the differentiation of ecotypes, especially for alpine and subalpine populations. The relation of respiration and photosynthesis to temperature in different populations of *Solidago virgaurea* are now being investigated at this institute. Preliminary results suggest that the temperature coefficient of the leaf respiration is ecotype specific to a certain extent and that it is smaller in mountain populations than in populations from lower levels.

The investigations started on the physiological and biochemical background of ecotype differentiation have indicated that a close control of the cultivation environment of the experimental plants is absolutely necessary. A phytotron, in which temperature, humidity and light are variable and controllable, will be used for the cultivation of ecotype material for continued studies.

Summary

Mountain and lowland populations of *Solidago virgaurea* cultivated under the same field conditions at Uppsala have been studied with regard to the

contents of flavonoid compounds and respiration intensity. Alpine and sub-alpine ecotypes have in autumn higher concentrations of anthocyanins and anthoxanthins in their leaves than plants from lower levels, whereas the latter have higher amounts of some saturated aromatic acids. At the same time leaf respiration was higher in mountain populations than in lowland populations.

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Induction Phenomena in Photosynthesis.

Experiments with *Polytrichum attenuatum*

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Several authors have shown the existence of a certain lag period in photosynthesis following the exposure of a plant to light, *i.e.*, the full rate of photosynthesis is not immediately attained. The length of the lag period varies from about one minute up to 20 to 30 minutes, and only then does the process attain its maximum rate under the given conditions. This accelerating phase until photosynthesis reaches its maximum rate is often referred to as the *induction phase*, and it has been shown to exist in unicellular algae as well as in more complicated organs of photosynthesis. A more detailed description of this phase with references to previous studies in this field is given by Steeman Nielsen 1942, Hill and Wittingham 1953, and Rabinowitch 1956.

Furthermore it has been shown that the increase in the carbon dioxide uptake of the plants during the initial part of the induction phase is not a steady one. On curves depicting dependence of photosynthetic CO₂-fixation on time (time curves) one or more peaks occur, particularly during strong illumination. These irregularities of the acceleration are often referred to as *induction phenomena*.

In most of the species studied, the carbon dioxide uptake increases rapidly during the first few seconds following the onset of illumination; there is then a sudden decrease with a subsequent second increase. Occasionally one or more additional peaks may be found in the time curve. However, these sub-

sequent fluctuations are usually less pronounced than the initial ones. This is true in general for all plants, although certain species have been found to give off carbon dioxide during the first few seconds of illumination. This is the case in *Chlorella* and *Protococcus*, while other green algae such as *Ulotrix*, *Hormidium* and *Stichococcus* show induction phenomena similar to those of other plants (van der Veen 1950).

The induction phenomena have been studied and described by Harder and Aufdemgarten 1938, Aufdemgarten 1939, and by van der Veen 1949 a, b, 1950. These authors have all been using measurements of the heat conductivity of the air in order to follow fluctuations in its carbon dioxide concentration. In the present work the induction phenomena in a species of moss were studied by means of a method essentially analogous to that used by van der Veen. The object was to determine the dependence of the acceleration irregularities on the carbon dioxide concentration, and, to a greater extent than has previously been done, their dependence on light intensity and temperature, to obtain a more detailed knowledge of their nature. Moss plants with their thin photosynthetic organs, rich in chlorophyll, were considered particularly appropriate for such experiments.

Material and Methods

Polytrichum attenuatum Menz. was chosen as experimental plant. The specimens used were collected in Rude forest (17 km. north of København) and were stored in the laboratory in covered glass dishes (height 10 cm., diameter 20 cm.). The plants were given an ample supply of water and were kept in a cool place in diffuse daylight.

The experimental arrangement is shown in Figure 1. Gas streams are passed from two steel cylinders (at A and A') containing compressed atmospheric air with 0.03, and slightly more than 3 vol% of carbon dioxide, respectively, through adjustable over-flows (B) and flow meters (D) into the mixing chamber (E). This is a hollow glass ball (diameter 56 mm.) with branched inlet tubes and an unbranched exit. The mixed air stream is guided into a humidifying chamber (F) in which it is saturated with water vapour by passing over a piece of moist gauze, one end of which is submerged in a 2 per cent solution of tartaric acid. The gas stream is then divided into two parts, one of which, the measuring stream, is passed through the plant chamber (G) and subsequently through another humidifying chamber into the measuring apparatus. The other one, the control stream, also passes through a humidifying chamber, but from there on it is taken straight to the measuring apparatus. Details are found in legend of Figure 1.

Besides the changes in the carbon dioxide content of the gas stream, it is often desirable to determine changes in the oxygen concentration; this may be done by diverting the measuring and control streams as shown in Figure 1 and using the diverted streams for oxygen measurements. Before these streams are taken to the

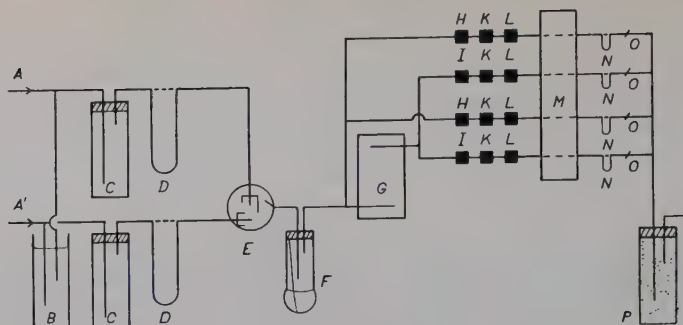


Figure 1. *Diagram of experimental arrangement.* A and A': inlet tubes from air cylinders, B: overflow, C: buffer flasks (for equalizing pressure fluctuations), D: flowmeters, E: mixing chamber, F: humidifying chamber, G: plant chamber, H: CO₂-absorption tube, I: tube with glass beads, K: humidifying chambers, L: drop traps, M: copper block with measuring ducts, N: flow meters, O: needle valves, P: cotton-filled buffer flask. Overflows and flowmeters D were adjusted in such a way that desirable carbon dioxide concentrations could be obtained in the mixing chamber.

measuring apparatus it is necessary that they be passed through tubes (H) containing a carbon dioxide absorbing substance (soda lime).

The gas streams are adjusted to identical rates of flow by means of flow meters (N) and needle-valves (O) inserted into each stream just behind the measuring apparatus. Finally all of the gas streams are united in one joint buffer bottle filled with cotton (P) to prevent as far as possible sudden fluctuations in the pressure of the surrounding air from influencing the flow rate. The measuring apparatus is surrounded by thermal insulating plates and placed inside two boxes, one inside the other, separated by air space.

1500 and 3000 watt incandescent lamps were used as light sources, and were placed in water baths, cooled by means of running water, to reduce the effects of infrared radiation. The plant chamber and the preceding humidifying chamber were placed in a thermostat bath in order to control the temperature of the experiment. A diaferometer built by the firm of Kipp & Zonen at Delft according to instructions by Noyons (1937) was used as measuring apparatus. This has four measuring ducts, two for measuring streams (CO₂- and O₂-measurements) and two for control streams. A platinum wire is suspended in each duct, and the wires of corresponding ducts are arranged in a Wheatstone bridge, each with its own galvanometer. A constant direct electric current of 100 milliamp. or more is passed through the platinum wires. Due to the changes in the composition of the air of the measuring stream and the resulting changes of its conductivity, a difference is caused in the temperature of the corresponding wires and thus in their resistance. The result is registered by a galvanometer deflection. The details have previously been described by Maas (1938), and Spierings, Harris, and Wassink (1952).

The current model of the apparatus is designed for measuring the respiratory quotient in humans and in animals. For photosynthetic measurements, where it is essential to utilize the full sensitivity of the apparatus towards carbon dioxide, it may be adjusted so that a galvanometer deflection of 1 mm. at 18°C corresponds

to a difference of 0.00060 per cent by volume of carbon dioxide in the two streams. In order further to enhance the sensitivity, the original galvanometer of the diaferometer was replaced by a more sensitive one (Kipp A 54), and also the distance from the galvanometer mirror to the scale was increased from the original 100 cm. to approximately 200. Finally the current through the platinum wires in the measuring ducts was increased from 100 milliamp. to 170 milliamp. The sensitivity was in this way improved to the extent that a galvanometer deflection of 1 mm. corresponds to 0.000077 per cent of carbon dioxide or to 0.77 ppm, all in terms of volume.

When measuring photosynthesis it would be of great interest to determine simultaneously the changes of oxygen as well as the changes of carbon dioxide contents of the gas streams. Hence much time was spent in an attempt to develop a method suitable for this purpose. However, the conductivity of pure oxygen differs by only about 5 per cent from that of atmospheric air, while the difference between the conductivity of carbon dioxide and atmospheric air is approximately 63 per cent (data of Laby and Nelson 1929). When oxygen measurements are carried out using Kipp's sensitive galvanometer (A 54), and with the distance from the galvanometer mirror to the scale increased to 600 cm., the apparatus gives a galvanometer deflection of 1 mm. for a change of 0.00073 per cent by volume, or 7.3 ppm in the oxygen content of the air. However, these changes cause great difficulties in the practical application of the apparatus, and it seems likely that an entirely different type of apparatus should be used for continual measurement of oxygen in photosynthesis (*cf.* Tietz 1954 and Hersch 1955).

The carbon dioxide concentration of the air in the steel flasks was determined by two methods. In the case of the weaker carbon dioxide concentration, 1 litre approximately of the air was transferred to a glass container and analyzed by means of an apparatus which was a modification of that described by Larsen (1949). The stronger carbon dioxide mixture was analyzed in a Haldane gas analysis apparatus as described by Boysen Jensen (1929). The carbon dioxide concentration of the gas leaving the mixing chamber of the photosynthesis apparatus (Figure 1, E) was controlled in the same way.

In each experiment 10 moss plants were used in the plant chamber. The plants were placed in the dark and the air stream (about 3 litre per hour) was adjusted to the carbon dioxide concentration to be used in the first experiment. This was started at the earliest after lapse of one hour, partly to allow the apparatus to reach temperature equilibrium, and partly to obtain a distinct appearance of the induction phenomena which are dependent upon the length of the previous dark period (Aufdemgarten 1939 a). After the first hour the galvanometer was read every 30 seconds. When the drift in the readings was only 2 to 3 mm. per minute the experiment proper was commenced by illuminating the plants. Thereafter the galvanometer deflection was read at first every 15 and subsequently every 30 seconds. The experiments were carried out in series. After the conclusion of the first experiment a dark period of at least one hour was inserted. During this period the carbon dioxide concentration, the light intensity, or the temperature was changed in due time, prior to the beginning of the second experiment, and so on until the series was completed. The slope of the drift curve was determined prior to and after each experiment, lasting 20 to 40 minutes. In case of failure to obtain identical slopes in the two measurements the experiment was discarded. The full course of the drift curve was drawn by means of the gradients measured and was used for correction of the time curve values.

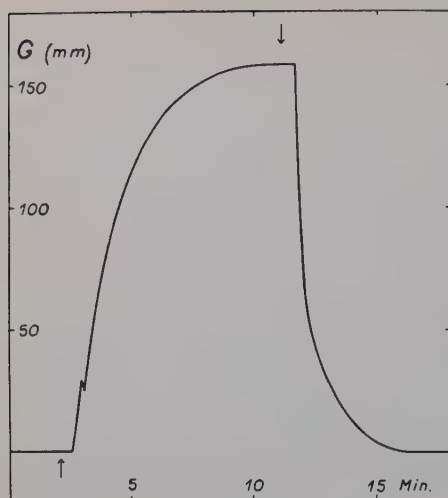


Figure 2. *Photosynthetic time curve for 10 moss plants in atmospheric air. (0.03 vol% CO₂). Light intensity 3000 lux. Temperature 20°C. Abscissa: time in minutes. Ordinate: galvanometer deflection in mm. ↑ Light on; ↓ Light off.*

Results

Aufdemgarten and van der Veen used carbon dioxide concentrations ranging from 0.3 to 4 per cent, *i.e.*, amounts 10 to 100 times those of the atmosphere. It would therefore appear to be of interest to show whether or not induction phenomena occur in atmospheric air with its 0.03 per cent of carbon dioxide. Figure 2 presents a curve from such an experiment, 10 moss plants being illuminated at 3000 lux in atmospheric air. In this case the time curve shows a slight but distinct peak approximately 30 seconds after the onset of photosynthesis.

The fact that in the present study photosynthesis in the time curves does not appear to set in until 30 to 60 seconds after the onset of illumination is due to inertia, caused by the experimental arrangement. The flow rate of the air is about 3 litres per hour, and depending upon the length of the connections of the experimental arrangement 30 to 60 seconds pass, before the gas from the plant chamber reaches the measuring ducts. A similar delay phenomenon may be observed at the end of each experiment when the light is turned off.

After it had proved possible to observe at least the primary induction stop in atmospheric air, series of experiments were planned to study the effect of the carbon dioxide concentration on the size of the peak. In Figure 3 are shown three such series all at 20°C. In the first one (Series A) the carbon dioxide concentration was kept at a constant value of 0.03 per cent while the light intensities of the individual experiments of the series were 1500, 3000, 6000, and 12000 lux. Between the individual experiments, dark periods

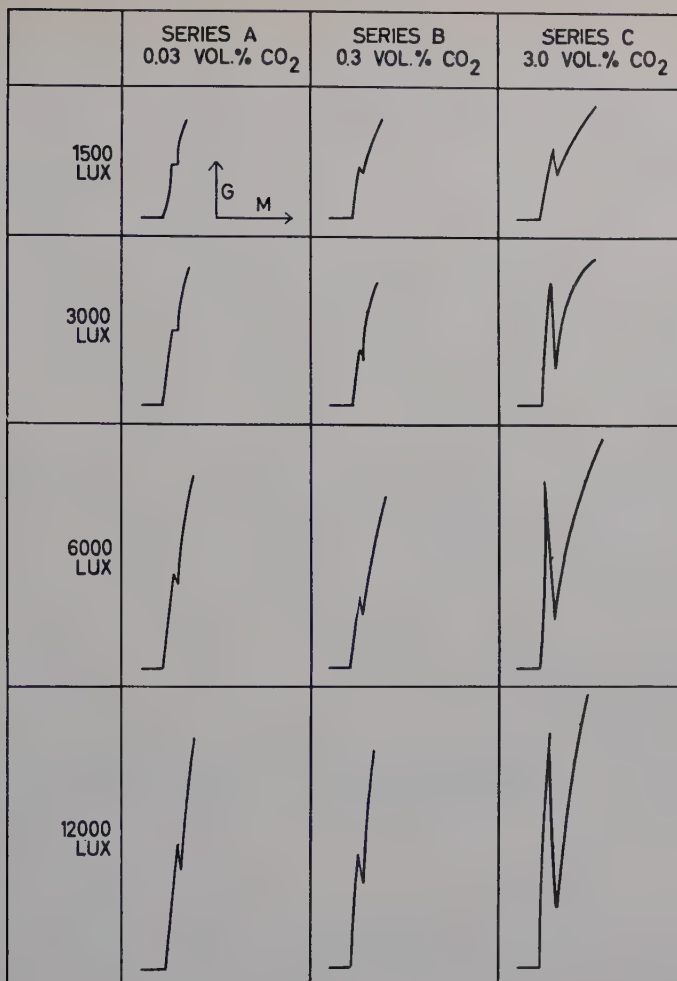


Figure 3. Induction phenomena at different carbon dioxide concentrations and light intensities. Abscissae M=4 minutes. Ordinates G=30 mm galvanometer deflection.

of 1 hour. In the two subsequent experiments (series B and C, figure 3) the carbon dioxide concentrations were 0.3 and 3.0 per cent respectively, but the over-all procedure was identical to that in the first series. For each series ten moss plants of as similar a size as possible, picked from one sample of equally treated material, were used.

In Figure 4 are shown two experiments, the first carried out at 12000 lux in 0.03, the second at 1500 lux in 3.0 per cent of carbon dioxide.

The curves of Figures 3 and 4 show very distinctly that at the atmospheric carbon dioxide concentration as well as at higher concentrations, and at both

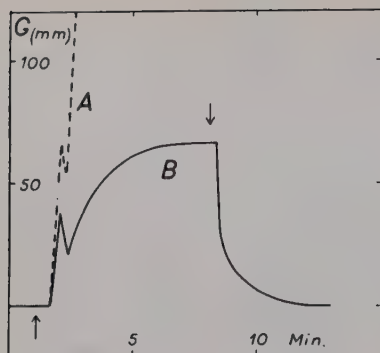


Figure 4. Induction phenomena at 0.03 % CO_2 and 12000 lux (A) and at 3 % CO_2 and 1500 lux (B). Abscissa: time in minutes. Ordinate: galvanometer deflection in mm.

low and high light intensities, a brief suspension of the carbon dioxide uptake occurs shortly after the onset of photosynthesis. In atmospheric air at 1500 lux the time curve shows only a slight shoulder, but as the light intensity increases it develops into a peak. In air mixtures with 0.3 and 3 per cent of carbon dioxide the time curve shows a peak in any case, but its size appears to increase with increasing carbon dioxide concentrations as well as with increasing light intensities. Harder and Aufdemgarten, as well as van der Veen, mention a second peak occasionally observed in the time curves of their plant material a few minutes after the tapering off of the first induction phenomenon. In the present *Polytrichum* experiments this second peak failed to occur, even at light intensities of up to 45000 lux and a carbon dioxide concentration of 3 per cent (Figure 5).

To elucidate the effect of temperature on the induction phenomenon in *Polytrichum*, several runs were made using constant light intensity and carbon dioxide concentration but with varying temperatures. The Figures 5 and 6 show time curves from an experimental series of this type. To favour the occurrence of the induction phenomenon an air mixture containing 3 per cent of carbon dioxide was used; the initial light intensity was 45000 lux. Curve A was obtained after the ten moss plants had been placed in the plant chamber in darkness for 90 minutes at 13°C . Subsequently the plants were darkened for one hour, the temperature rising to 30° during the first 20 minutes. Hereafter the curve assumes the shape shown in curve B. To make sure that the plants had in no way been injured by heating, they were cooled once more to 13° and after a total of two and a half hours in the dark (necessary for the cooling process) curve C was obtained.

These experiments show the initial uptake of carbon dioxide in *Polytrichum* to depend on the temperature to a great extent. The induction peak appears very distinctly on the time curve when the experiments are carried

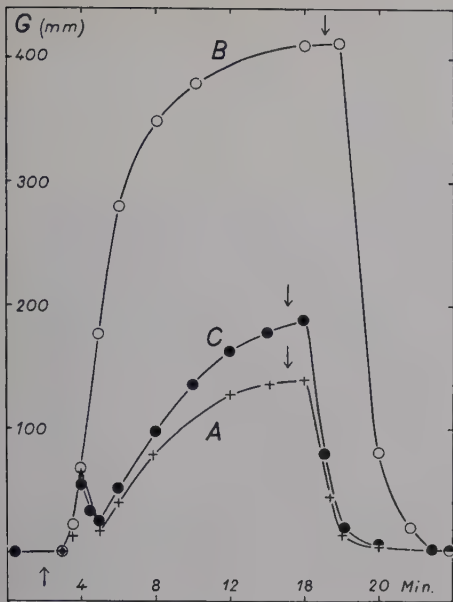


Figure 5.

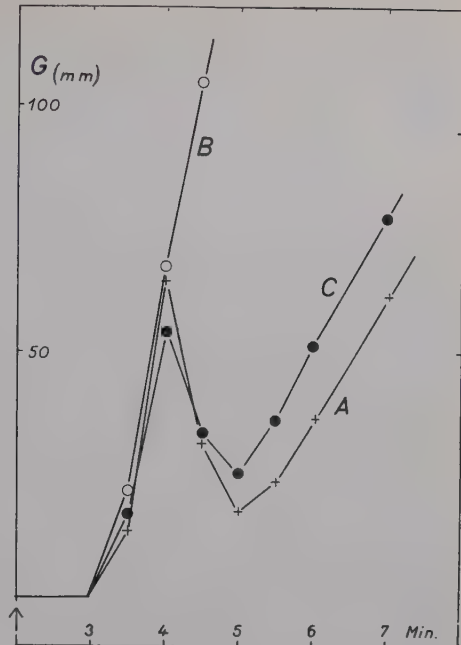


Figure 6.

Figure 5. *Photosynthetic time curves for 10 moss plants in 3 % CO₂. Light intensity 45000 lux. Temperatures: curve A 13°C., curve B 30°, curve C 13°. A, B, and C in three subsequent experiments. Abscissa: time in minutes. Ordinate: galvanometer deflection in mm.*

Figure 6. *Detail of Figure 5 at a larger scale.*

out at 13° but disappears almost completely at 30°. If the plants are then cooled to 13° the phenomenon reoccurs to the same extent as before.

The results of the present experiments concerning the initial uptake of carbon dioxide in *Polytrichum attenuatum* immediately following exposure to light may be summarized as follows.

a) Photosynthesis begins with a vigorous uptake of carbon dioxide, which under certain conditions ceases after 25 to 60 seconds, only to be reassumed after a similar period, but at a somewhat smaller rate. After 5 to 20 minutes the uptake of carbon dioxide reaches its maximum value at the light intensity, temperature, and carbon dioxide concentration concerned, and continues constantly at this value. When the light is removed the uptake of carbon dioxide decreases abruptly, but does not altogether cease until a few minutes later.

b) The mentioned cessation of the initial uptake of carbon dioxide depends

upon a number of external factors. Increasing carbon dioxide concentrations and light intensities produce an increasing peak in the photosynthetic time curve. With increasing temperatures the peak decreases and disappears at 30°

Discussion

Calvin and Massini 1952 considered the uptake of carbon dioxide in photosynthesis to be a carboxylation of one molecule of ribulose-1,5-diphosphate (RuDP). They assumed that the product of the carboxylation were subsequently split up into two molecules of 3-phosphoglyceric acid (PGA) which were reduced during the process of photosynthesis. Quayle, Fuller, Benson and Calvin 1954 were able to reproduce the carboxylation reaction using plant extract, and RuDP is now generally recognized to be the carbon dioxide acceptor of photosynthesis.

Thus the presence of a certain quantity of RuDP must be a prerequisite for photosynthesis to be able to start simultaneously with the onset of illumination. Further, an additional, initially accelerating later steady formation of this carbon dioxide acceptor is required during the further course of the photosynthesis process.

Through paper chromatography and radioautography Bassham, Shibata, Steenberg, Bourdon and Calvin (1956) were able to show that the contents of RuDP and PGA in *Scenedesmus* depend to a great extent on the illumination or non-illumination of the plants. According to their studies the concentration of the carbon dioxide acceptor decreases upon the transfer of the plants from light to dark to a level no longer demonstrable with the methods used, in a matter of about 25 seconds. On reverting from dark to light the content of RuDP slowly increases to the steady state level over a period of 8 to 10 minutes.

Contrary to RuDP the concentration of PGA increases by darkening to more than twice the content at the steady state level in the light. During the subsequent 20 minutes in the dark the content of PGA decreases to a fairly constant value, approximately 50 per cent higher than the light value. With repeated illumination a slight increase takes place momentarily, followed by a decrease to a value slightly lower than that ordinarily found in the light and which is reached approximately one minute after the onset of illumination.

The curves drawn by Bassham *et al.* recording the fluctuations in the contents of RuDP and PGA in *Scenedesmus* on transfer of the plants from light to dark and vice versa resemble to a certain extent the curves representing the present results. On the basis of the knowledge acquired in recent

years concerning the reaction mechanism of photosynthesis, it seems reasonable to explain the observed induction phenomenon in the following way:

At a low concentration of carbon dioxide (0.03 per cent) the plants utilize an amount of RuDP already present as carbon dioxide acceptor directly following the onset of illumination. In the case of low light intensities the photosynthetic intensity increase nearly linearly with time until the maximum value is reached. However, a slight break occurs in the uptake of carbon dioxide after about 30 seconds probably due to a momentary lack of RuDP. This break becomes more pronounced with increasing light intensities. At higher light intensities, however, the acceleration of photosynthesis is likewise greater. The initial part of the time curve increases more steeply and the reservoir of the carbon dioxide acceptor present may therefore be expected to decrease more rapidly, resulting in an increasingly pronounced peak formation in the time curve (Figure 3 A).

At higher carbon dioxide concentrations (0.3 and 3.0 per cent) the carboxylation of the RuDP present under normal conditions will proceed more quickly. This in turn causes photosynthesis to accelerate more rapidly, but at the same time the momentary deficiency in the carbon dioxide acceptor will be so much the greater (Figures 3 B and C).

It is a well known fact that the peak in the time curve does not occur if the light is switched on immediately after an illumination period, but only if the dark period preceding is of a duration of about 8 to 10 minutes (Aufdemgarten 1939 a, van der Veen 1949 a). The smooth acceleration (non occurrence of the peak) might be explained as resulting from the higher concentration of PGA which is found soon after darkening. If light is turned on during this period, reduction of PGA will immediately start and give rise to a production of RuDP at a rate presumably sufficient to counteract the decrease in the carbon dioxide uptake causes the occurrence of the peak in the time curve.

It appears from Figures 2, 4, and 5 that the rate of uptake of carbon dioxide decreases rapidly immediately after the light has been turned off, although zero value is not reached until 3 to 4 minutes later. This delay is hardly attributable to inertia of the apparatus, but it may possibly be explained on the basis of the assumption that within this period of time a certain, though decreasing, amount of carbon dioxide acceptor will still be present. Although the curves published by Bassham *et al.* depicting the concentration of RuDP in *Scenedesmus* show that this falls to zero within 30 seconds in the dark, the possibility of different conditions in *Polytrichum* exists.

The absence of the induction phenomenon at higher temperatures (Figures 5 and 6) may be due to two different reasons. One reason, probably the main one, is the fact that all of the temperature dependent dark reactions of

photosynthesis proceed at a greater rate at higher temperatures, and that therefore the carbon dioxide acceptor will be regenerated more rapidly. The second reason is the likewise greater rate at higher temperatures of all the respiration processes. These processes may also add to the accumulation of available RuDP since this compound is a product of the so-called hexose-monophosphate-shunt (Horecker, Smyrniotis and Seegmiller 1951 and Axelrod, Bandurski, Greiner and Jang 1953).

The amounts of RuDP and PGA measured by Bassham *et al.* were determined as the amounts of those compounds containing ^{14}C added in the form of $^{14}\text{CO}_2$ during previous illumination. Therefore the plants may well contain larger or smaller amounts of these substances originating from respiratory processes.

The hypothetical explanation of the induction phenomena here presented further seems to support the previously ventilated idea (Rabinowitch 1956) that the entire induction phase could be interpreted as an accelerated formation of RuDP. However, the offered explanation can only be considered as a tentative one, it is by no means as yet proved that RuDP production is the only limiting factor during the induction phase and the first irregular part of the time curve.

Summary

The induction phase and the induction phenomenon during the initial uptake of carbon dioxide in photosynthesis is studied in the moss *Polytrichum attenuatum* Menz. by means of the diaferometer method. The dependence of the induction phenomenon on carbon dioxide concentration, light intensity, and temperature is determined. The time curve is in agreement with corresponding curves for higher plants, but has only one peak 30 to 60 seconds after the onset of photosynthesis. The size of the induction peak increases with increasing carbon dioxide concentrations and light intensities, but decreases with increasing temperatures. It disappears completely at 30°C only to reoccur at lower temperatures.

An attempt is made to explain the induction phenomenon in relation to recent studies concerning the amounts of carbon dioxide acceptor (ribulose-1,5-diphosphate) and 3-phosphoglyceric acid present in plants in the light and in the dark respectively.

For the present work grants were received from the Carlsberg Foundation (the diaferometer) and from the Danish State General Research Foundation (additional equipment and technical assistance). The diaferometer technique was studied under Professor E. C. Wassink, Agricultural University, Wageningen, The Netherlands, where the preliminary experiments were also made. The author is indebted to Professor Wassink and to Dr. Spierings for their hospitality and assistance.

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Concentration of Carbon Dioxide and Rate of Photosynthesis in *Chlorella pyrenoidosa*

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1. Introduction

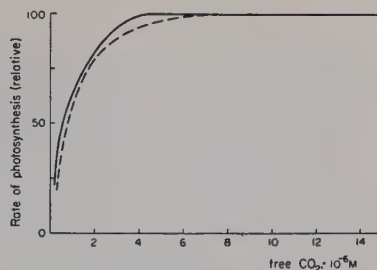
The classical investigations of Warburg 1919 and of Emerson and Green 1938 did apparently give a clear picture of the relation between CO_2 -concentration and photosynthesis of the unicellular plankton alga *Chlorella pyrenoidosa*, an alga which has been used in thousands of investigations in a multitude of laboratories all over the world. During the last ten years, however, confusion has arisen concerning both the nature of the carbon sources utilized in *Chlorella*-photosynthesis and the degree to which this process is influenced by the CO_2 -concentration.

Warburg used mixtures of 0.1 M NaHCO_3 and Na_2CO_3 and a manometric technique. Full saturation of photosynthesis was found at a concentration of free CO_2 of about 90×10^{-6} M, half saturation at 3×10^{-6} M. According to Emerson's and Green's measurements, half saturation was found at the same concentration, full saturation was, however, found at 8×10^{-6} M free CO_2 .

The latter investigators admitted an amount of CO_2 to a vessel containing a suspension of *Chlorella pyrenoidosa* at pH 4.5, and followed the disappearance of CO_2 from minute to minute by a differential manometer. They could then compute the concentration of the CO_2 remaining at any given time and the corresponding rate of photosynthesis.

Whereas some recent investigations have given results according to which

Figure 1. *The relative rate of photosynthesis in Chlorella pyrenoidosa as a function of the concentration of free CO₂. Full drawn line according to a pH-technique (Steemann Nielsen 1955), dashed line according to infrared CO₂-analyses (Whittingham 1954).*



a very high concentration of free CO₂ seems necessary for saturation of photosynthesis in *Chlorella*, other experiments to be described below indicate just the opposite.

In recent series of investigations made in Cambridge and in Copenhagen the dependence on carbon dioxide concentration in the photosynthesis of *Chlorella* has been studied. Half saturation of photosynthesis was found at an even lower concentration of free CO₂ than that given by the earlier investigators. — *i.e.* between about 0.5 and about 0.8×10^{-6} M.

In Cambridge Whittingham 1952 used the infra-red gas analyser method, according to which the photosynthetic rate is measured as a function of decreasing CO₂ content in a closed system; pH was 4.6. Steemann Nielsen 1955 used a technique according to which the photosynthetic rate was measured as a function of increasing pH. In Figure 1, two curves, in principle identical, obtained by means of these two methods are presented.

At the time when Warburg made his experiments it was practically a dogma that only free CO₂ could be used as a carbon source in the photosynthesis of aquatics. In recent years, however, it has been shown through the work of Ruttner, Arens, Steemann Nielsen, and Österlind, that many — but not all — aquatic species are able to utilize bicarbonate ions as well as free CO₂. Österlind 1949 has given a survey of the literature. The ability is most easily shown in leaves of phanerogamic aquatics where the utilization of bicarbonate is accompanied by a release of OH⁻-ions from the upper side of the leaves (*cf.* Arens 1933, Steemann Nielsen 1947).

Österlind 1949 has indisputably shown that the planktonic green alga *Scenedesmus quadricauda* is able to utilize bicarbonate ions directly as a carbon source; his investigations concerning *Chlorella pyrenoidosa* did not give any indication of a direct utilization of bicarbonate ions in this species. The same result was found by Briggs and Whittingham 1952.

According to recent investigations by Gaffron (unpublished but quoted by Rabinowitch 1956 pp. 1888—1890) *Chlorella pyrenoidosa* is, however, able to utilize bicarbonate. A technique of measuring the photosynthetic rate as a function of a decrease in pH was employed. This technique was worked

out by Rosenberg 1954, who measured the dependence of the photosynthesis of *Chlorella* on the concentration of free CO_2 . According to his measurements half saturation of photosynthesis is found at a concentration of free CO_2 of $10^{-4} M$, i.e., at a concentration more than 100 times as high as that found in Cambridge or Copenhagen. Of course, the dependence on CO_2 concentration may vary in *Chlorella*. In Copenhagen we have occasionally found that slightly higher concentrations are necessary than those presented in Figure 1. The order of magnitude, however, is the same.

Rosenberg's technique may be regarded as admirable in many respects. Unfortunately, however, at least one detail has been overlooked. Rosenberg worked out his technique with the aim of measuring rapid changes in photosynthetic rates. He employed a continuously recording glass electrode for measuring the pH. In order to obtain rapid changes, extremely dense algal suspensions were employed — 10 ml. packed algae per liter. There are several drawbacks to the use of dense suspensions. Only one will be mentioned here, since a criticism of the technique was given by Steemann Nielsen 1955 (see also p. 178). According to the curves (Rosenberg's Figure 3) 120 seconds only are necessary to produce a change in pH from 7.00 to 7.65. If the rate of photosynthesis had not decreased within this range of pH, the change should have taken only about 90 seconds. From pH 7.00 to pH 7.65 the concentration of free CO_2 decreases to less than one fourth. Rosenberg must have tacitly assumed that the speed of the change in CO_2 -concentration has no influence on the rate of photosynthesis.

Two years previously (1952) Briggs and Whittingham have shown that an induction time in photosynthesis is needed when transferring *Chlorella* from a high to a low concentration of free CO_2 . The duration of the induction time may be up to two hours. Briggs and Whittingham interpreted their findings in terms of the production of a photosynthetic depressant which is removed by some photochemical process other than photosynthesis at a low concentration of carbon dioxide. The findings of Briggs and Whittingham concerning induction time in Warburg buffers have been fully corroborated in Copenhagen. In 2 mM KHCO_3 , however, the induction time is much shorter.

Rosenberg used a 2 mM bicarbonate solution in his experiments. According to Rabinowitch 1956, Gaffron was able to alter the shape of the " CO_2 curve" by increasing the bicarbonate concentration. In 0.1 M bicarbonate the curve becomes practically flat, down to values as low as 30×10^{-6} moles free CO_2 /liter. According to Gaffron (Rabinowitch) the increasing bicarbonate concentration increases the "residual" rate attributable to bicarbonate. According to this, a considerable rate of photosynthesis should be possible in *Chlorella pyrenoidosa* with bicarbonate as a direct carbon source.

2. Experimental Part

An interpretation of Gaffron's observations quite different from that of Rabinowitch may be made. By augmenting the bicarbonate concentration the rate of increase in pH with time decreases. The rate of the decrease in concentration of free CO_2 with time is thus slowed down, permitting the cells to make an easier adjustment to the continually decreasing CO_2 concentrations.

The same slowing down of the alterations may of course be effected by lowering the density of algae but using the same bicarbonate concentration (2 mM).

Experiments were therefore made in which 4 different *Chlorella pyrenoidosa* strains were used: a) an Emerson strain, b) a strain from Professor Harder, Göttingen, c) a strain from Dr. Rodhe, Uppsala, d) a strain isolated in Copenhagen. The algae were cultured in Davies' medium at 25°C , using Drechsel gas washing bottles, aerated by 5 % CO_2 . They were illuminated by fluorescent light at about 7,000 lux at the front of the Drechsel bottles.

After centrifugation and washing, the cells were re-suspended in 2 mM KHCO_3 . In most cases free CO_2 was bubbled through for a very short period in order to decrease the pH somewhat. By means of a siphon the suspension thereupon was distributed into about thirty 12 ml. bottles with glass stoppers. These bottles were attached to a big rotating wheel in an illuminated (fluorescent light) water bath at 25°C . Illumination at the front of the bottles was about 8,000 lux. The mean light intensity for the algae varied according to the density of the suspension. In the thinner suspensions employed — 0.04 ml. of packed cells per liter, the light absorption in the bottles was only minimal. Already at a density of 0.5 ml. per liter, the mean light intensity was sufficiently reduced to influence the rate of photosynthesis. In the diluted suspensions the rate of photosynthesis per volume of algae was the same as that stated by Rosenberg 1954, who used extremely high light intensities in order to counteract the light absorption in his very dense suspensions. Using Emerson's strain, we normally obtained a photosynthetic rate of 15×10^{-6} moles CO_2 per min. per ml. of packed algae. After the start of the experiments, bottles were withdrawn from the water bath at intervals, and the pH was determined with a glass electrode under standardized conditions. At low pH values the apparatus was adjusted by using a phosphate buffer at pH 6.80, at high pH values by using a borate buffer at pH 9.78. The rate of photosynthesis and the concentration of free CO_2 at any pH value was determined using the Table 1 of Rosenberg 1954.

Figure 2 shows an experimental series with strain d, using a concentration of 0.5 ml. of packed cells per liter. This concentration is one twentieth of

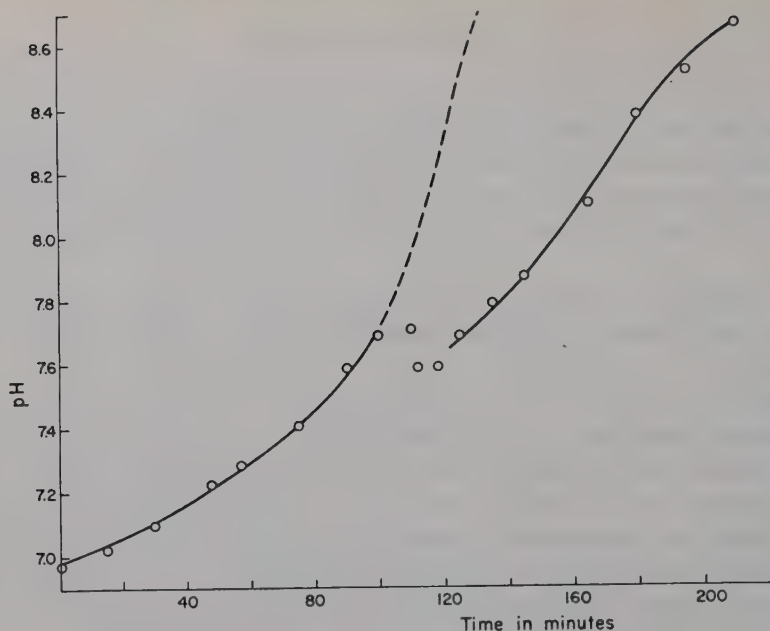


Figure 2. Variation of pH as a function of time in 12 ml. bottles containing a suspension of 0.5 ml. packed cells of *Chlorella pyrenoidosa* (strain d) per liter 2 mM KHCO_3 , adjusted initially by free CO_2 to pH 7.0. 8,000 Lux, 25°C. The dashed line indicates how the curve would have continued, if the rate of photosynthesis had not decreased.

that used by Rosenberg. The rate of photosynthesis remained constant up to pH 7.7. Thereupon for nearly half an hour photosynthesis seemed to have stopped completely; it started again subsequently, but at a much lower level. According to this series, saturation of photosynthesis is found at about 90×10^{-6} M free CO_2 , i.e., a concentration less than one sixth of that stated by Rosenberg.

Similar series with the same strain in the same concentration yielded somewhat similar results. The complete stopping of photosynthesis at about pH 7.7 was, however, not so distinct as appears in Figure 2. In the Emerson strain such a stop has not been observed.

Figure 3 illustrates an experimental series made with Emerson's strain at a concentration of 0.04 ml. of packed algae per liter, i.e., a concentration less than one twohundredth of that used by Rosenberg. As the rate of increase in pH (because of the very diluted suspension) is very small, it was necessary to divide the suspension in two parts, one of which was started at pH 7.0 (after addition of free CO_2), the other at pH 8.1.

The rate of photosynthesis, as measured from the increase in pH with

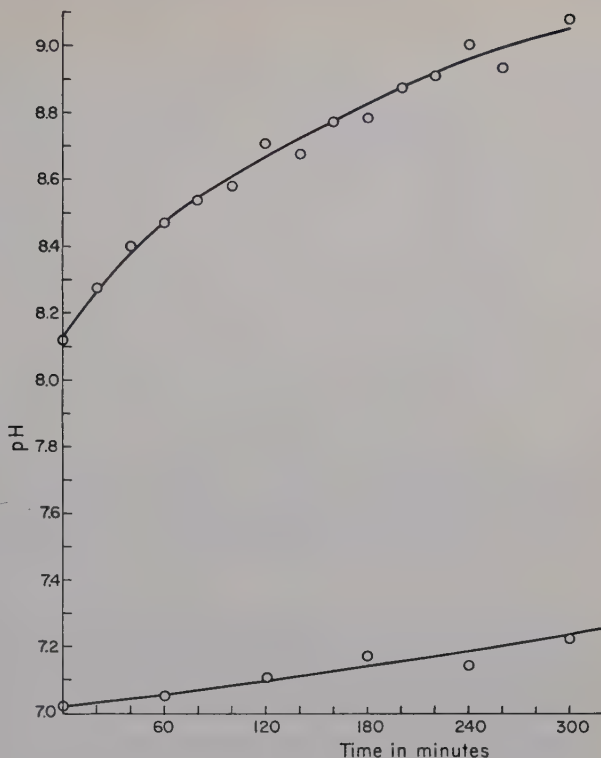


Figure 3. Variation of pH as a function of time in 12 ml. bottles containing a suspension of 0.04 ml. packed cells of *Chlorella pyrenoidosa* (strain a) per litre 2 mM KHCO_3 . A part of the bottles were filled with a corresponding solution adjusted initially by free CO_2 to pH 7.0 — lower curve, 8,000 Lux, 25°C.

time, now remained constant up to about pH 8.4, where the concentration of free CO_2 is $17 \times 10^{-6} M$. In other similar series saturation of photosynthesis was found decisively below $10 \times 10^{-6} M$ free CO_2 . There is no reason for presenting these series. It is possible with other techniques to obtain a better accuracy of the measurements; cf. Figure 1.

It is, however, beyond all doubt that the apparently very high CO_2 concentrations, necessary for saturation of photosynthesis, according to Rosenberg, are due to the too rapid changes in CO_2 concentration making it impossible for the algae to complete their "induction" periods.

The statement by Gaffron in Rabinowitch 1956 of bicarbonate assimilation in *Chlorella pyrenoidosa* is therefore hardly correct. The statement by Briggs and Whittingham 1952 that there is no evidence that bicarbonate ions are utilized by this species can in principle be agreed to.

The question arises, however, whether bicarbonate ions are completely unable to penetrate into *Chlorella* cells. A priori it would seem somewhat unlikely that this should be so. Some species — like, e.g., *Scenedesmus quadricauda* — are extremely permeable to bicarbonate. A very slight penetra-

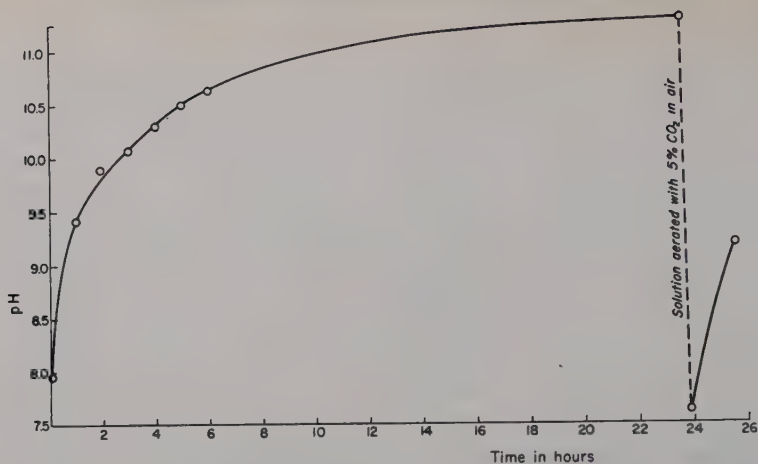


Figure 4. Variation of pH as a function of time in 12 ml. bottles containing a suspension of 0.5 ml. packed cells of *Chlorella pyrenoidosa* (strain a) per liter 2 mM KHCO_3 . 8,000 Lux, 25° C. After 23 1/2 hours the pH was adjusted to 7.6.

tion rate would be of no practical importance for photosynthesis under normal conditions. On the other hand, once bicarbonate has penetrated into a cell it must be used in photosynthesis, because of the presence of the enzyme carbonic anhydrase.

Experiments of different kinds were therefore made. The curve in Figure 4 shows an experiment in which the pH was followed for 24 hours. The normal bicarbonate concentration was employed — 2 mM. The relatively high algal density — 0.5 ml. of packed algae per liter — was used in order to shorten the duration of an experiment, 24 hours in pure KHCO_3 being considered the limit for such a treatment. During the 24 hour period the pH increased to 11.2. It has not been possible to obtain a higher value for pH in such experiments with *Chlorella*. In the determination of the pH at a value of about 11 the maximum error was ± 0.1 . By a short treatment with an air flow enriched with 5 % CO_2 , the pH decreased to 7.6; immediately the pH started to increase again. The algae had not suffered much by staying for some time at a pH of above 11. It may be mentioned that true bicarbonate-users such as *Myriophyllum spicatum* may cause an increase in the pH until they are damaged by it (Steemann Nielsen 1947.).

Thus at pH 11.2 at a concentration of only 4×10^{-9} M free CO_2 , the compensation point between photosynthesis and respiration is found. Without an influence attributable to bicarbonate ions, the concentration of which at pH 11.2 is 0.2 mM, it seems very unlikely that photosynthesis should equal respiration. According for example to Gabrielsen 1949, the carbon dioxide

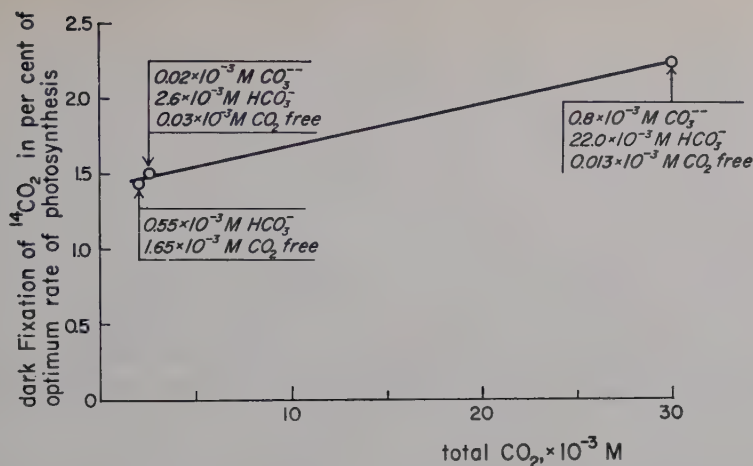


Figure 5. Rate of dark fixation of $^{14}\text{CO}_2$ (percentage of optimum rate of photosynthesis) as a function of the concentration of total CO_2 . 25°C , *Chlorella pyrenoidosa* (strain b).

compensation point in leaves of terrestrial plant is found at about $4 \times 10^{-6} \text{ M}$. There is no reason to expect a difference by a factor 1000 between the carbon dioxide compensation point in *Chlorella* and in leaves of terrestrial plants.

Since a direct investigation of the compensation point in *Chlorella* in solutions of only free CO_2 may involve some complications because of the extremely low concentrations (no gas-analyser technique was available), another method of investigating possible penetration of bicarbonate ions into *Chlorella* cells was attempted instead.

It is well known that fixation of CO_2 takes place in the dark. In rapidly growing cultures of *Chlorella pyrenoidosa* this dark fixation is about 1 % of the optimum light fixation for experiments with a duration of about 2 hours. Dark fixation of ^{14}C was therefore investigated in solutions with varying concentrations of CO_2 and HCO_3^- . The technique described by Steemann Nielsen 1952 was used. Figure 5 illustrates these experiments. The concentration of total CO_2 is given as the abscissa. At the different points from which the curve is constructed, the concentrations of CO_2 , HCO_3^- and CO_3^{2-} are presented.

At a concentration of about 2.5 mM total CO_2 the dark fixation is the same whether the main part of CO_2 is in the form of free CO_2 or bicarbonate ions. At a concentration of 30 mM the dark fixation has increased by 50 % although the concentration of free CO_2 is considerably less than in the experiments with a concentration of 2.6 mM total CO_2 . This is possible only on the supposition that bicarbonate ions penetrate into the *Chlorella* cells. It must, however, be remembered that the rate of dark fixation of carbon-14 com-

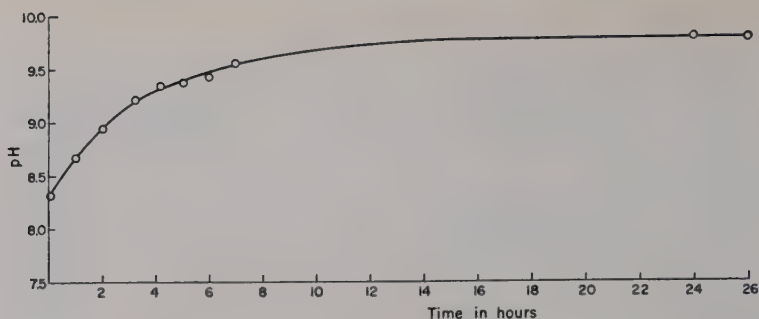


Figure 6. Variation of pH as a function of time in 12 ml. bottles containing a suspension of 0.5 ml. packed cells of *Chlorella pyrenoidosa* (strain a) per liter 10 mM KHCO_3 . 8,000 Lux. 25° C.

pared with the rate of the fixation in light due to photosynthesis is very low. The rate of bicarbonate penetration into the cell need therefore be only very slow.

Hence it may be concluded that a direct utilization of bicarbonate ions takes place during photosynthesis in *Chlorella*. The rate is, however, so low that it is of importance only under special conditions.

Treatment of *Chlorella* for extended periods in Warburg buffers has a depressing effect on the rate of photosynthesis. The effect is already observable at much lower concentrations of carbonate-bicarbonate than in those used by Warburg. Figure 6 shows a series of experiments with *Chlorella*. The increase in pH with time is presented in a 10 mM buffer. At the start of the experiment the rate of photosynthesis as measured from $\frac{dpH}{dt}$ was identical with that in a 2 mM buffer. During 24 hours in the 2 mM buffer the pH increased to 11.2 (*cf.* Figure 4) whereas the pH in the 10 mM buffer increased only to 9.75 in 26 hours. The experiments presented in Figures 4 and 6 were made simultaneously with algae from the same culture. If the rate of photosynthesis in the latter buffer had continued to be the same as in the one first mentioned, the pH should have increased to 10.5. The rate of photosynthesis must hence decrease with time in a 10 mM potassium carbonate-bicarbonate buffer. Steemann Nielsen 1947 showed that the corresponding decrease in the phanerogamic aquatic *Myriophyllum spicatum* when photosynthesizing in the same solution is much more rapid.

Warburg buffers have often been used in studies of photosynthesis in aquatics. Results obtained under such experimental conditions must often be regarded with some reservation.

3. Appendix

In an article published recently Rosenberg 1957 has answered the criticism made by Steemann Nielsen 1955 concerning the methods used by him for measuring in *Chlorella* the dependence of photosynthesis on the concentration of carbon dioxide, measurements which apparently showed that high CO_2 concentrations were necessary for optimum rate of photosynthesis.

Rosenberg rejects the criticism, his main point being that his methods are free from systematic errors. But he admits finally that his results are valid only for the conditions appertaining the adaptation time which is required for algae to make efficient use of carbon dioxide at low pressures. Whereas Rosenberg notes all the other points in the criticism, this special point, the last one in the criticism, appears to have escaped his attention. Steemann Nielsen wrote 1955 p. 329: "Finally the rather long induction times necessary in photosynthesis when going from a high to a low CO_2 concentration — see Briggs and Whittingham (1952) and Whittingham (1952) — must seriously influence the results of experiments when the CO_2 concentration is changing rapidly. Collectively these objections do not substantiate Rosenberg's results which differ so much from the results obtained by other investigators".

As Rosenberg and the present authors now seem to agree in principle there should be no need for further discussion. However, one very important detail in Rosenberg's methods must be referred to. Rosenberg writes 1957 p. 827: "The possibility of heating effects exaggerated by the use of dense suspensions, as suggested by Steemann Nielsen, was indeed eliminated in the original experiments by the use of infrared absorbing filters between the light source and the sample". This is not correct. In an experiment at 60,000 lux comparable in practically all respects with those made by Rosenberg's transient method, the temperature of the algae increased from 24° to 28°C within five minutes. The infrared absorbing filter ON 20 (Change) was used in addition to a water layer five cm. thick. It must be kept in mind that the filter only absorbs the infrared light. Since only an insignificant part of the visible light is transformed due to photosynthesis into chemical energy in a very dense culture most of the energy (about $0.4 \text{ cal./cm}^2 \cdot \text{min.}$) is transformed into heat.

Rosenberg's methods may be of considerable importance for studies of photosynthesis. A *sine qua non* is, however, that high algae densities are to be avoided and that an effective temperature regulation of the experimental vessels is applied. Finally the experimental water must be stirred.

4. Summary

Using a modification of Rosenberg's transient method of 1954 and very thin algae suspensions it is shown that *Chlorella pyrenoidosa* is able to photo-

synthesize at optimum rate at very low concentrations of free CO_2 . This is in agreement with most earlier investigations. It is shown that Rosenberg's discordant results are due to the dense suspensions used by him, which primarily have the effect that CO_2 concentration changes too rapidly and does not permit the necessary induction time in photosynthesis when changing from a high to a low CO_2 concentration.

The view by Gaffron in Rabinowitch 1956 concerning bicarbonate assimilation in *Chlorella pyrenoidosa* is not corroborated. The experiments are explained in another way.

It is shown nevertheless that bicarbonate ions are able to penetrate into *Chlorella* cells. The rate of the penetration is, however, so low that it is of importance for photosynthesis only under special conditions.

Treatment of *Chlorella* for extended periods in Warburg buffers has a depressant effect on the rate of photosynthesis. The effect is observable at much lower concentrations than are found in the usual 0.1 *M* carbonate-bicarbonate buffers.

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Evidence for a Sexual Hormone in *Allomyces*

By

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Sexual reproduction in several algae and fungi has been shown to be controlled by specific chemical substances — hormones. The evidence was comprehensively and critically reviewed by Raper in 1952 (1) and again in 1957 (2). In the last review Raper clearly stated the challenge this field of study has for the investigator: extensive efforts to define chemically even one of these hormones have so far been unsuccessful.

The hormonal control observed in *Allomyces* and described in this report is simple compared to the complex interrelations that exist in *Achlya*. Evidence will be presented that a hormone is produced during female gametogenesis (here used to include the cleavage of the protoplast into female gametes and their subsequent release as swarmers) which attracts motile male gametes to the female gametes. The progress made towards isolation and identification of the hormone indicates a stability and molecular size compatible with eventual complete chemical characterization. The investigation was facilitated by a crude but effective bioassay and through the use of male and female strains derived from the normally hermaphroditic species of *Allomyces* included in the subgenus *Euallomyces*.

It is of interest that Raper in 1952 (1) anticipated many of the results set forth in this paper. He wrote "In considering these many cases of hormonal activity in the algae, it is of parenthetical interest that such a correlative mechanism has not as yet been found in any fungus having gametic copulation, although the similarity between formation, morphology, and behavior of planogametes in aquatic fungi and algae would indicate a common type

of regulatory mechanism in the two cases. That the correlative mechanism has not been found in fungi possibly results from the fact that in none of the forms reproducing by differentiated gametes is there sexual dimorphism coupled with the capacity of indefinite vegetative propagation; it is therefore impossible to secure large clonal masses of fungal planogametes of known sexual affinities. The discovery, or creation in the laboratory by means of genic mutations, of heterothallic strains of *Allomyces*, etc., would in all probability be shortly followed by description of a hormonal coordinating mechanism in planogametic fungi."

Materials and Methods

The organisms used consisted of the natural Burma 1Da strain of *Allomyces macrogynus* and of interspecific hybrids of *A. macrogynus* and *A. arbuscula*. The hybrids differ from their hermaphroditic parents in being essentially unisexual. The life history, morphology, systematics, and cytology of *Allomyces* have been studied and described in detail (3, 4, 5). Similarly, numerous facets of their physiology have been investigated (6 to 13). A brief summary will be adequate here. The organisms consist of two isomorphic, independent generations each of which grows without limit on the surface of nutrient agar with rhizoids penetrating into the agar. The mycelia bear numerous reproductive structures which are readily scraped free of the culture. On the gametophyte are found the orange, male gametangia and larger, colorless, female gametangia produced in approximately equal numbers. The gametangia tend to be produced in pairs, with the female terminal in *A. macrogynus* and the male terminal in *A. arbuscula*. The gametangia from such an hermaphroditic plant can be placed in water and separated laboriously into male and female with a Pasteur pipette.

On the sporophyte are found the thin-walled, colorless, mitosporangia (in which mitotic nuclear divisions precede the formation of diploid mitospores) and thick-walled, pitted, brown, meiosporangia (in which meiotic nuclear divisions precede the formation of haploid meiospores). The former, when placed in water, quickly release motile mitospores which develop into new sporophytic plants. Since heavy suspensions of mitospores are readily prepared, they are the choice for inoculating liquid media when sporophytic growth is desired. The meiosporangia, on the other hand, are dormant for periods of a few days to several weeks after their formation, depending on the strain and the cultural conditions. When mature and placed in water, they germinate releasing motile, haploid, meiospores which develop into new gametophytes. When germination is good, the meiosporangia can be freed of other sporophytic material (mycelium and mitosporangia) by heating to 30°C for 24 hours. The suspension of meiospores subsequently obtained can be used to inoculate liquid cultures when gametophytic growth is the objective.

In liquid agitated culture, both the gametophytes and the sporophytes initially grow almost completely vegetatively in the form of soft, spherical pellets. As the medium becomes unfavourable for further growth the pellets develop reproductive structures all over their surface. This is particularly true when growth is limited by a nutrient deficiency rather than the development of a low pH.

In the present study, the plants were grown in a medium in which the supply of sugar was regulated so as to become depleted before the pH dropped excessively. Upon completion of growth, the plants were filtered into small baskets made of monel metal 60 mesh screen. After washing the plants with water or a dilute salt solution (DS solution) the basket with its plants was placed in a small petri dish containing water. During the next one to three hours swarmers were released. Upon removing the basket with its plants a clear suspension of swarmers was left in the dish.

The nutrient agar used in both slants and plates contained 1.0 g. K_2HPO_4 , 0.5 g. $MgSO_4 \cdot 7H_2O$, 15 g. soluble starch, 4 g. yeast extract, 20 g. agar, and 1000 ml. double distilled water (3). Medium B of Machlis (8) containing 0.005 M KH_2PO_4 , K_2HPO_4 , and $(NH_4)_2HPO_4$, 0.0005 M $MgCl_2$ and $CaCl_2$, 0.15 mg./l. thiamine HCl, 0.1 g./l. L-methionine and trace elements was used in liquid cultures with the glucose concentration reduced to 0.2 per cent and with the inclusion of 14.7 mg. L-glutamic acid per liter. Slants and plates were incubated at room temperature which varied between 22 and 25°C while the liquid cultures were grown at 25°C on a shaking machine (8). DS solution consists of the major inorganic nutrients of Medium B diluted ten times.

Results

The presumption of a hormone in *Allomyces* was based on a simple observation undoubtedly made by almost everyone who has worked with the organisms. When male and female gametangia are placed in water, male gametes cluster around undischarged female gametangia. Subsequently, of course, the males seek out and fuse with the released female gametes. The clustering of the male gametes about female gametangia is in itself unproductive, except that the males are then in the area into which the female gametes will move upon leaving the gametangia. The clustering suggested that a substance was diffusing outward from the gametangium. In early crude tests, male and female gametangia were separated with Pasteur pipettes. The females, while in the process of gametogenesis, were embedded in hot agar at approximately 60°C. When bits of this agar were placed in hanging drops in the presence of male gametes, the male gametes were observed to cluster about the bits of agar. A similar response was obtained when approximately equal numbers of both male and female gametangia were embedded in the agar. The presence of male gametangia did not interfere with the reaction nor did the presence of female gametes interfere with the response of the males. These initial tests were done with the Burma 1Da strain of *A. macrogynus*. It was obvious from the beginning that the study would be greatly facilitated were it possible to obtain unisexual strains and to develop some quantitative method of recording the reaction rather than attempting to estimate the degree of clustering about an irregular piece of agar.

The Selection of Unisexual Strains

In the course of their study of the inheritance of gametangial arrangement, Emerson and Wilson (5) made the incidental observation that the proportion of male to female gametangia borne by the progeny of certain interspecific crosses deviated markedly from the normal ratio of 1 : 1. The authors kindly made available meiosporangia preserved from their cross S108 in which the Ceylon 1 strain of *A. arbuscula* ($n=16$) served as the female parent and the Burma 1Da strain of *A. macrogynus* ($n=28$) supplied the male gametes.

The meiosporangia were germinated and the meiospores plated out on nutrient agar. Three isolates were selected: M-1 (92 % ♂), M-6 (87 % ♂) and F-1 (98 % ♀). These isolates have retained their maleness or femaleness through several months of successive transfers. A variety of crosses between these isolates, including selfing and parthenogenesis, were made in an effort further to increase the unisexuality. Femaleness was increased only from 98 to 99 per cent while maleness was increased from 92 to 99 per cent. The female isolates saved and now carried in stock cultures were: F-1, the original female isolate; F-2 (99.1 % ♀) resulting from parthenocarpic development of F-1 female gametes; and F-3 (97.1 % ♀) resulting from selfing of the F-1 isolate. The male isolates continued in culture were: M-1 and M-6, the original male isolates; M-2 (97 % ♂) and M-3 (96 % ♂) resulting from parthenocarpic development of M-1 female gametes; M-4 (98 % ♂) and M-5 (99 % ♂) resulting from a cross of M-2 (♀ parent) and M-3 (♂ parent); and M-7 resulting from the selfing of the M-1 isolate.

The female plants obtained are parthenocarpic, a characteristic inherited from the *A. arbuscula* parent. For reasons to be discussed later it was preferable to have nonparthenocarpic female plants, whose unfertilized female gametes would fail to grow instead of giving rise to sporophytic plants. It seemed possible that distorted sex ratios might be obtained by crossing two strains of *A. macrogynus* differing in chromosome number. India B4 ($n=14$) was reciprocally crossed with Burma 1Da ($n=28$). Although the meiosporangia were aged extensively germination was always poor. A number of gametophytes were obtained, however, but their sex ratio was always approximately 1 : 1. No further efforts were made to obtain nonparthenocarpic female plants.

The Bioassay

The bioassay depends on the attraction of male gametes to the lower surface of a membrane which has in contact with its upper surface a solution of the hormone. The reaction can be recorded photographically or, by the use of a grid in the eyepiece of a microscope, as the number of gametes

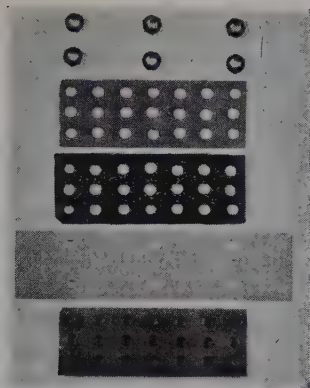


Figure 1.

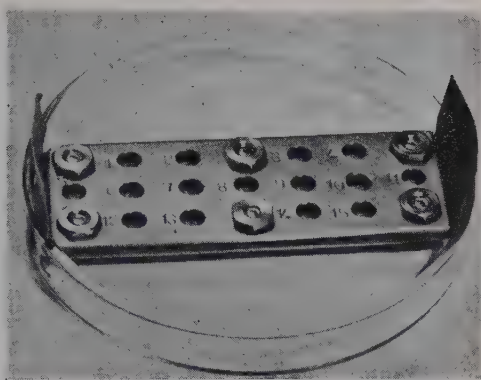


Figure 2.

Figure 1. *The unassembled bioassay apparatus.* From bottom to top: the lower plate threaded with the six bolts; the membrane (cardboard substituted here for visibility); the rubber gasket; the upper steel plate; and the nuts for locking apparatus together.

Figure 2. *The assembled bioassay apparatus in place in the petri dish.*

per unit area of the membrane. The parts of the apparatus are illustrated in Figure 1. It consists of two stainless steel plates and a rubber gasket, each $3.2 \times 2.8 \times 0.15$ cm. and penetrated by holes 5 mm. in diameter, a sheet of dialyzing membrane, and six sets of stainless steel nuts and bolts. The bolts are threaded into the lower steel plate. The heads of the bolts hold the plate approximately 3 mm. above the bottom of the petri dish in which the assembled apparatus is placed (Fig. 2). The membrane is placed on top of the lower plate (with appropriate holes to permit passage of the bolts), followed by the gasket, the upper steel plate, and finally the nuts which are tightened just enough to make each hole above and below the membrane watertight. The apparatus is then placed in a selected flat petri dish containing a suspension of male gametes of such depth as to cover the lower steel plate. Bubbles underneath the membrane are removed by suction through a bent tube as is also any liquid in the cells above the membrane. Test solutions are then placed in the cells and observations made at a magnification of approximately 100. If photomicrographs are to be taken it is necessary to fill completely the cells and cap them with a cover glass.

The suspensions of male gametes, prepared as follows, were almost completely free of extraneous materials such as empty and undischarged gametangia, mycelial fragments and bits of agar. Pieces of acid-treated, high wet-strength, filter paper (such as is used in chromatography), 1.5 cm. square, were placed on the surface of nutrient agar plates. Each paper was

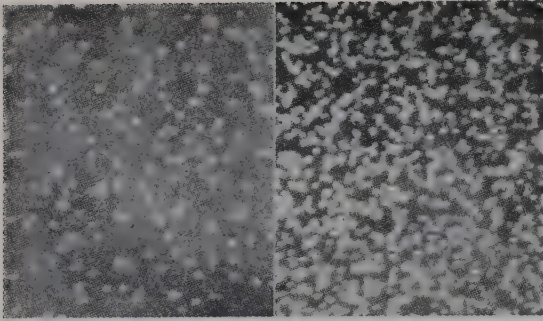


Figure 3. *Male gametes attracted to the membrane when the cell contained water (left) and hormone solution (right).*

inoculated with two small mycelial blocks of male plant and growth permitted to proceed for about a week at 25°C . The paper was then lifted from the plate and placed bottom side down in a wire basket which was, in turn, put in a 6 cm. petri dish containing 8 ml. of DS solution. The dish was placed on a shaker moving just enough to wash the solution gently back and forth. During the first hour the DS solution was changed at 15 minute intervals and then left without change for a second hour. Upon removing the basket, a clear suspension of male gametes was left in the dish containing up to 1.1×10^6 male gametes per ml. After determining the density with a haemocytometer (12), the suspension was diluted to 5×10^5 gametes per ml.

The reaction is rapid. Within five minutes gametes can be observed congregating against the membranes of the cells containing the hormone. Initially the gametes against the membrane are very motile and counting them is impossible. After approximately 40 minutes the gametes tend to stay in position on the membrane and the number per unit area remains constant for approximately 30 minutes (Figure 3). After that they slowly leave the membrane presumably because of diffusion of the hormone throughout the suspension of gametes. Although the 15 cells per plate all feed into a common liquid reservoir no interaction between cells has been observed. A water control cell adjacent to a cell containing the hormone has a very low count throughout the test while the hormone-containing cell has a high count. The areas of membrane counted were the same in any one test but varied from time to time. Usually this area was approximately 0.0005 mm^2 but accurate records were not kept; hence data are reported simply as the number of males per unit area. Typical results are illustrated by Table 5.

The Preparation and Assay of Crude Hormone Extracts

Extracts of the hormone were prepared by two methods, one using female plants grown on nutrient agar and the other using plants grown in liquid

Table 1. *The hormonal activity of extracts obtained from plants grown on nutrient agar.*

Type of plant	Male gametes per unit area
Female (F-2).....	10
Male (M-2)	3
Sporophyte (Burma 1Da) ...	3
Sporophyte (F-2 selfed)	2

medium. Nutrient agar in 9 cm. petri dishes was inoculated with mycelial blocks at the center and at four equally-spaced points about the perimeter of the plates. Maximum growth was completed in approximately a week. The plate was then flooded with 10 ml. DS solution and allowed to stand for one or two minutes before scraping the surface clean with the edge of a glass microscope slide. The gametangia and DS solution were then decanted into a small petri dish in which gametogenesis proceeded for the next 90 minutes. The preparation of the extract was completed by filtration through a Seitz filter. These extracts contain, in addition to the hormone, material dissolved from the agar, both nutrients and excreted metabolic products, soluble materials from the disrupted mycelia, and metabolic products released during gametogenesis.

Plates of female strain F-2, male strain M-2, Burma 1Da sporophyte, and a sporophyte obtained by selfing male strain M-1 were used as sources of extracts prepared as described above. As shown in Table 1, only the extract from the female plants was active. Nutrients from the agar, excreted metabolic products, and materials released from disrupted hyphae had no activity since these substances were common to all four preparations. The specificity of the hormone was further confirmed by testing a variety of substances for activity. Completely negative results were observed with solutions at pH 7.0 ± 0.1 of the following: indoleacetic acid, gibberellic acid, and kinetin at 1.5 and 10 mg. per liter; adenine, cytosine, guanine, uracil, thymine, xanthine, ribonucleic acid, and deoxyribonucleic acid at 500 mg. per liter (guanine, adenine, and xanthine were incompletely dissolved at this concentration); and yeast extract, casein hydrolysate, vitamin-free casamino acids, L-glutamic acid, bactotryptone, and soluble starch at 1.5 and 10 g. per liter.

In the first experiment it was assumed that the hormone was produced during female gametogenesis. This was confirmed using female plants grown in liquid culture (see below for details of culture and extract preparation).

The medium in which the plants had grown, with the pH adjusted to the original value of 7.0, was usually inactive. Occasional indications of slight activity were readily correlated with a slight amount of premature gametogenesis in the culture flasks. In contrast, the DS solution in which these same

Table 2. *The effect of various temperatures on the hormonal activity of crude extract.* The extracts were sterilized by passage through a Seitz filter and stored at the temperatures indicated for 30 days. The sample at 95°C evaporated to dryness and was made up to volume with water. Tests 1, 2 and 3 were made at the same time and refer to three different cells of the bioassay apparatus.

Temperature of storage °C	Male gametes per unit area			
	Test 1	Test 2	Test 3	Average
— 20	18	20	19	19
22	16	19	24	20
70	13	18	23	18
95	19	18	20	19
DS solution	—	4	—	4

plants underwent gametogenesis was always very active. It was also shown that extracts from female plants were inactive towards female gametes.

The preceding experiments indicate that during female gametogenesis a specific substance is released which attracts male gametes. According to Raper's definitions (2) this is a sexual hormone. The hormone would appear to have a low molecular weight since it passes rapidly through a dialyzing membrane.

While still working with the crude extracts, the stability of the hormone was assessed. It was found very early that the extracts could be taken to dryness in a vacuum oven at 40°C without complete loss of activity (partial loss was not ascertainable). In a more critical test aliquots of an extract (sterilized by filtration through a Seitz filter) were stored for 30 days at —20, 22, 70, and 95°C. The latter aliquot evaporated to dryness and was made up to volume with distilled water. The results (Table 2) show no loss of activity at any of the temperatures used. If, however, unsterilized extract is kept at room temperature for a day or two, the activity is lost presumably as the result of microbial destruction of the hormone. A further indication of stability is the retention of activity when the hormone is chromatographed. No loss was observed when the hormone was retained for up to four days on dried paper exposed to light at room temperature. Finally, an extract at pH 7.0 kept in boiling water for one hour was not inactivated. In contrast, all activity was lost if the extract was made approximately 1-N with either KOH or HCl and then heated for the hour.

The degree of stability found was encouraging and the loss of activity with acidic or basic hydrolysis suggested that an ester was involved. Procedures were therefore developed to obtain substantial amounts of extract less contaminated with extraneous materials than the extracts prepared from plants grown on nutrient agar.

The Preparation and Fractionation of Extracts from Plants Grown in Liquid Medium

The ideal inoculum for gametophytic plants to be grown in liquid medium is a suspension of meiospores. However, meiosporangia known to yield female plants had such poor germination that meiospore suspensions of adequate concentration could not be obtained. An alternate but less elegant procedure was developed. The female gametophyte was grown on nutrient agar and the entire culture or a sector, agar and plant, placed in a blender cup with approximately 20 ml. DS solution and then fragmented for one to two minutes at maximum speed. The 125 ml. Erlenmeyer flasks containing 50 ml. of medium were each then inoculated with 1.0 ml. of the suspension. Even though viable female gametes were present which developed parthenocarpically into sporophytic plants, the bulk of the growth was female plants developing from the fragments of gametophytic mycelium. At the end of a week the plants, by then covered with female gametangia, were separated from the medium by pouring the contents of the flask into a wire sieve basket. The plants were then washed for 10 seconds in running distilled water and finally placed in a 6.0 cm. petri dish with 10 ml. distilled water for gametogenesis to take place. After three hours the basket with the plants was removed and the remaining solution freed of gametes by passage through a Seitz filter. The solution was concentrated approximately 30 times *in vacuo* at 40°C thus yielding a solution yellow in color with a pH of 6.1—6.3.

This solution was chromatographed for amino acids (14), sugars (14), fatty acids (15), and substances visible under ultraviolet light (14). Four amino acids only were detected of which one was considerably more con-

Table 3. *Location of hormonal activity and of the amino acid contaminants on ascending chromatographs prepared with various solvent systems.* The concentrated solution was applied as a horizontal strip. The paper was subsequently divided into five horizontal strips which were eluted in water and the water solution tested for activity. An intact part of the chromatogram was sprayed with ninhydrin to detect the amino acids. The chromatography was according to Aronoff (14).

Solvent system	Position	
	Of activity	Of amino acids
BuOH/HAc/H ₂ O (4—1—1)	Upper one-fifth	Lower five-tenths
Butyric acid/BuOH/H ₂ O (2—2—1)	Upper one-fifth	Lower six-tenths
Ether/HAc/H ₂ O (13—3—1)	Upper one-fifth	Lower two-tenths
95 % EtOH/NH ₄ OH (100—1)	Upper one-fifth	Lower five-tenths
80 % Phenol	All levels	All levels
Cresol (water-saturated)	Upper three-tenths	Lower seven-tenths
Collidine (water-saturated)	Destroyed	Lower four-tenths

Table 4. *Distribution of hormonal activity on a chromatogram.* The solution was applied as a horizontal strip and chromatographed ascendingly with ether/HAc/H₂O (13—3—1). The paper was then divided into 5 horizontal strips (upper part of table) or 10 (lower part of table) which were eluted into water and the water solution then tested for activity. The strips are numbered from the bottom of the chromatogram to the height reached by the solvent front.

Horizontal strip number	Male gametes per unit area
5	19
4	2
3	3
2	3
1	5
10	40
9	15
8	6

centrated than the others. When these amino acids were eluted into water and tested for activity the results were negative. Further, the same amino acids were obtained from sporophytic plants grown in liquid. These concentrated solutions from sporophytic plants have never shown any activity. Thus, the only known substances detected were eliminated as the hormone.

The concentrated hormone-containing solution prepared with female plants was chromatographed ascendingly using the various solvents listed in Table 3. After the papers were thoroughly dried they were cut into horizontal strips and each strip eluted overnight in 1.0 ml. of water. The detailed results of one such run are given in Table 4. When separation of the hormone was achieved, the hormone always travelled in the solvent front. No system was found in which the hormone was between the origin and the front. It

Table 5. *The effectiveness of ether in extracting the hormone from a water solution.* The unconcentrated solution of hormone was extracted with six successive portions of ether. The table also shows the reproducibility of the bioassay method.

Solution	Test 1		Test 2		Test 3		Test 4		♂'s Average
	Cell ¹ No.	♂'s ²	Cell No.	♂'s	Cell No.	♂'s	Cell No.	♂'s	
DS solution	(1)	5	—	—	(8)	8	(15)	8	7
Water solution of hormone ...	(2)	25	(5)	30	(9)	25	(12)	25	30
Water after ether extraction	(3)	6	(6)	8	(10)	10	(13)	10	8
Ether extract	(4)	25	(7)	30	(11)	30	(14)	40	32

¹ Cell number in apparatus. The numbering scheme was 1—4 for the top row from left to right, 5—11 for the middle row left to right, and 12—15 for the bottom row left to right (see Figure 2).

² Male gametes per unit area.

appeared, therefore, that the hormone was lipoidal in nature. The best separation of hormone from the amino acids known to be present was obtained with the ether/Hac/H₂O system.

Since a definitive chromatographic separation was not attained, solvent extraction methods were tried. When the hormone solution, prior to concentration, was extracted with six successive portions of ether, each equal to one-third the volume of the solution, all activity and no amino acids passed into the ether (Table 5). When the ether solution was evaporated to dryness, a yellow, fatty material was found. If a very small amount of this material on the end of a 1.0 mm. glass rod was swirled in water for three hours, the water became highly active although no discernible solution of the material occurred.

Discussion

This investigation was initiated with the hope that if evidence for the existence of a sexual hormone in *Allomyces* were obtained, its isolation and chemical composition could be profitably pursued. It seems clear that a specific substance is produced during female gametogenesis which attracts male gametes to the female gametes. The existence of a sexual hormone in a gametic fungus is thereby demonstrated. The substance is relatively stable to heat, light, and various solvents and appears to be of relatively low molecular weight. It should, therefore, be possible to isolate and identify the hormone.

Summary

Evidence is presented that a hormone which attracts male gametes to female gametes is produced and excreted during female gametogenesis by a watermold in the subgenus *Euallomyces*. The hormone can be boiled for one hour at neutrality but is inactivated or destroyed when similarly heated in 1-N KOH or HCl. It can be extracted from an aqueous solution with ether.

The hormone diffuses rapidly through a dialyzing membrane, a characteristic which indicates a small molecular size and which forms the basis for the bioassay. The assay method was facilitated by the use of unisexual strains, approximately 99 per cent pure, which were obtained by genetic selection.

This research was done in the Department of Botany, University of California, Berkeley, California with the support in part of a research grant (G 1291) from the National Science Foundation. I am indebted to Mary Esta Ashton and Edwin Risser for their excellent technical assistance.

The writing of this report was done during part of my tenure as a Guggenheim Fellow. I wish to express my sincere appreciation to Professor Nils Fries who provided me with supplies and facilities in the Institute of Physiological Botany, University of Uppsala, Uppsala, Sweden during the period of the fellowship.

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A Chemical Study of the Neomorphosis Induced by Glycine in *Oenanthe aquatica*

By

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It was recently reported by one of us (Waris 1957) that an excess of glycine (0.1—0.4 %) in aseptic nutrient solutions containing sucrose (1 %) has a striking morphogenetic effect on *Oenanthe aquatica*. In the beginning the seedlings grow normally, but within 3 to 4 months they become morbid and seem to be on the point of dying. Later on, however, a new mode of reproduction sets in, giving rise to a very strange neomorphic plant. From the root tips of the original morbid seedlings minute grains become detached and develop into simple plants with strap-shaped leaves, resembling dwarf *Vallisnerias*. In the same solution in which the original seedlings turn brown, the neomorphs exhibit a fresh green colour and reproduce vigorously from initially colourless epidermal outgrowths detached spontaneously from the leaves. The roots are reduced to short tiny threads. In many cases the young plants first form two cotyledon-like leaves, thus resembling normal seedlings. The neomorph grows submerged and never becomes more than a few centimetres long, but complicated groups are formed by branching.

When transferred to nutrient solutions containing sucrose but no glycine, the abnormal development continues for several months, but eventually some of the plants commence to form normal aerial shoots. Thus no irreversible change of the genotype has taken place.

It is evident that external glycine more or less upsets the intermediary metabolism of the plant. The present study was undertaken to find out how this disturbance might be reflected in the concentrations of the soluble inter-

mediates of carbohydrate and protein metabolisms. Quantitative fractionation into soluble and insoluble nitrogen, and a qualitative chromatographic study of these fractions as well as of the soluble sugars are reported in the present paper.

Methods

Cultivation. The plants were grown from seeds sterilized with 10 % hydrogen peroxide. Erlenmeyer flasks holding 750 ml. were provided with 250 ml. of a complete mineral nutrient solution containing 1 % sucrose, and, in some series, 0.1 or 0.4 % glycine. At the start the pH was about 6.

Preparation of trichloroacetic acid (TCA) extracts. The plants were washed with cold water and rapidly weighed after the external water had been dried off between layers of soft cellulose tissue. A representative sample was separated for dry weight determination (100°C overnight).

20 g. of fresh material (containing ca. 19 g. of water) was crushed in a mortar in 19 ml. of ice-cold 16 % TCA. The suspension was rapidly centrifuged, the insoluble residue was washed once with ice-water, centrifuged, dried, and weighed. It was used for determinations of insoluble nitrogen and the preparation of a protein hydrolysate.

The clear supernatant was freed from TCA by 4 h. continuous ether extraction and was slowly run through a small column of Amberlite IR-120 (6×50 mm.) which was then washed with 20 ml. of water. The combined eluates were concentrated to dryness *in vacuo*, made up to 1.0 ml with 10 % propanol in water, and their content of free sugars investigated by paper chromatography.

The adsorbed free amino acids were displaced from the resin by 50 ml. of 1 N NH_4OH , this was distilled off *in vacuo*, the residue was made up to 1.0 ml. with 10 % propanol and its content of free amino acids was studied by paper chromatography.

Protein hydrolysates. 500 mg. of dried insoluble cell residue+10 ml. of 6 N HCl were sealed into a heavy-walled test tube and kept at 108°C for 24 h. After cooling, the hydrolysate was distilled to dryness *in vacuo*, a few ml. of water was added, and the humin was removed by filtration. The clear hydrolysate was repeatedly evaporated to dryness, dissolved in water three times for removal of HCl, and finally made up to 1 ml. with 10 % propanol.

Hydrolysates of the amino acid extracts. 0.5 ml. of the final extract+0.5 ml. of conc. HCl were sealed in an ampoule and kept for 24 h. at 108°C. HCl was removed and the residue made up to 0.5 ml. as above.

Nitrogen determinations were made by the standard micro-Kjeldahl method, the distillation being performed in the apparatus designed by Parnas and Wagner.

Paper chromatography was made 2-dimensionally on Whatman No. 4 paper (60×60 cm. sheets) in large tanks as described earlier (Miettinen 1954).

A. Sugars

Solvent I: *n*-butanol: propionic acid: H_2O =500 : 270 : 350 (v/v), 18 h.

Solvent II: phenol: H_2O =1,000 : 368 (w/v), 18 h. in an NH_3 atmosphere (100 ml. 0.5 % NH_4OH on the bottom of the tank).

Colour reagents: Ammoniacal AgNO_3 and/or aniline hydrogen phthalate.

Different mixtures of 20 common mono- and oligosaccharides were run as controls.

B. Amino acids

Solvent I: *n*-butanol: acetic acid: $H_2O=630:100:270$ (v/v), after standing for 48 h. upper phase used. Run for 36 h. in an atmosphere containing traces of HCN.

Solvent II: as above+traces of HCN in the atmosphere, 12—16 h.

Colour reagent: ninhydrin.

Different mixtures of 40 natural amino acids were run as controls.

Absorption spectra of TCA extracts were determined by the Beckman quartz spectrophotometer using 1 cm. quartz cells, at 20°C.

Results

The colour of the TCA extract of the normal plant and that of the neomorph, grown on a glycine medium, is strikingly different. The normal plant gives a bright pink extract, whereas that of the neomorph is pale brownish yellow (Figure 1). It is evident that the neomorph does not contain the red pigment, absorbing at the region of 550 $m\mu$, which is present in the normal plant.

Qualitative (Figures 2—4) and quantitative (Table 1) determinations of different fractions of the cell constituents reveal little difference, if any, in

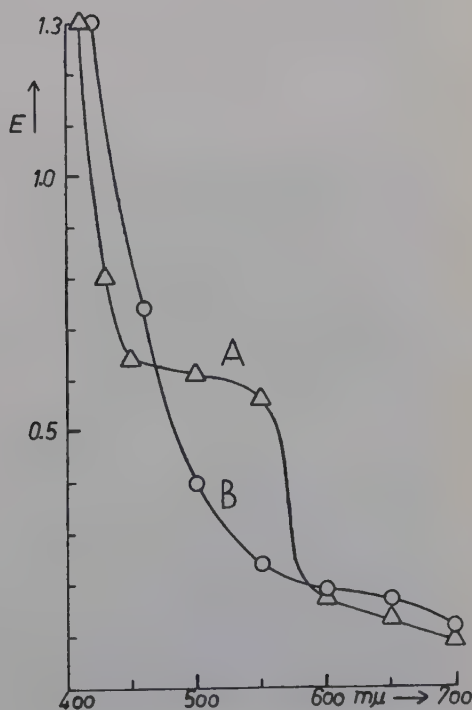


Figure 1. Absorption spectra of trichloroacetic acid extracts of *Oenanthë aquatica* grown on normal (A) and glycine-containing (B) media.

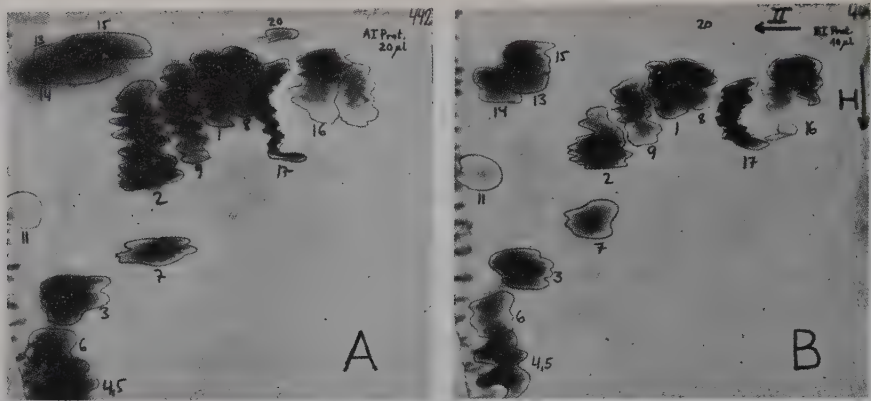


Figure 2. Protein amino acids of *O. aquatica* grown on normal (A) and glycine-containing (B) media.

1 = glycine	5 = isoleucine	9 = threonine	15 = lysine
2 = alanine	6 = phenylalanine	11 = proline	16 = aspartic acid
3 = valine	7 = tyrosine	13 = histidine	17 = glutamine acid
4 = leucine	8 = serine	14 = arginine	20 = cysteine

the quantity and amino acid composition of the protein fraction. The same usual protein amino acids are present in both types in about the same concentrations (Figure 2).

Both the soluble (Figure 3) and the insoluble (Table 1) carbohydrate contents of the normal plant are definitely higher. A larger amount of insoluble

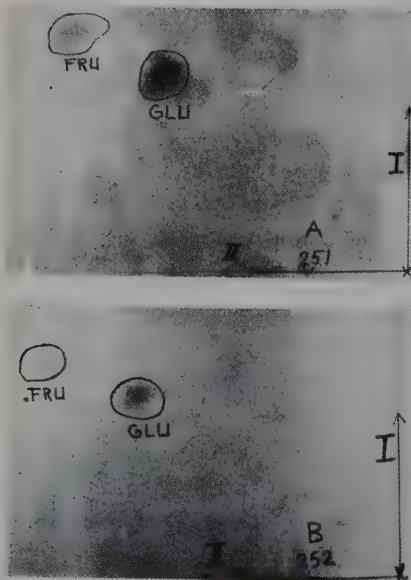


Figure 3. Paper chromatograms of free sugars of *O. aquatica* grown on normal (A) and glycine-containing (B) media.

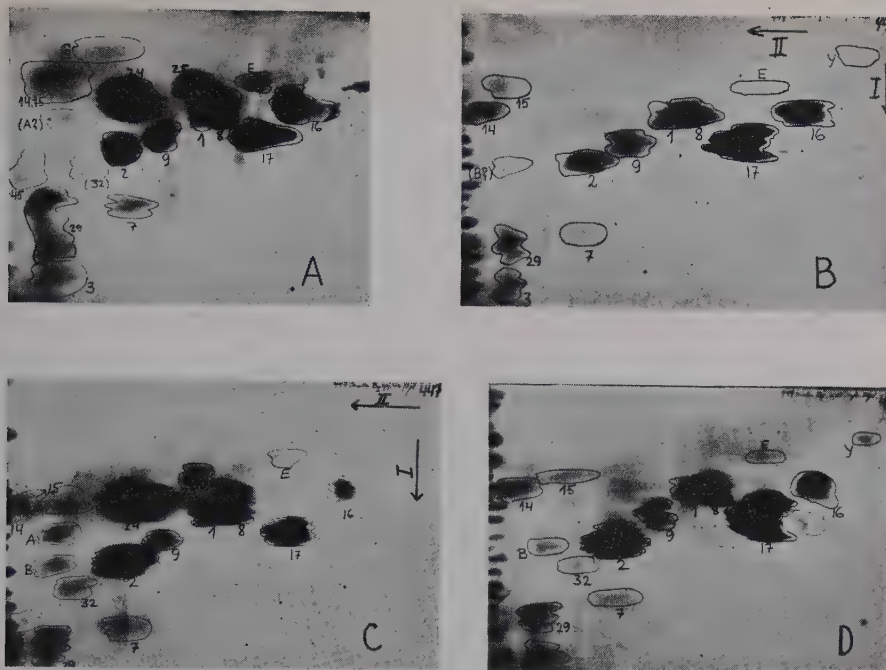


Figure 4. Free amino acids of *O. aquatica* grown on normal (A, B) and glycine-containing (C, D) media. 0.15 mg N on each chromatogram. A and C unhydrolyzed, B and D hydrolyzed.

24 = glutamine

29 = γ -aminobutyric acid

45 = ethanolamine

25 = asparagine

32 = β -alanine

A, B, G, E, Y = unknown compounds

carbohydrate in the normal plant is understandable, since the bulk of it consists of highly differentiated stem tissue, rich in structural cellulose.

A lower concentration of free sugars in the neomorph probably reflects an inadequate synthesis of carbon structures. Glucose and fructose are found in both cases, their level in the neomorph being about $1/2$ — $1/3$ of the normal.

The most profound difference is to be seen at the level of the soluble nitrogen, which is more than 10 times higher in the neomorph (Table 1). Qualitatively, the difference is not so great — *e.g.* the relative amount of glycine itself is about the same in both cases — although a few minor differences are apparent on paper chromatograms (Figures 4 A and C). The neomorph contains 2 unknown spots, A and B, which are not present in the normal plant. Of these, A is evidently a peptide, since it is decomposed on hydrolysis, but B a new amino acid, since it is stable on hydrolysis and not identical with any amino acid available in this laboratory. With the solvent system used, it takes about the same position as sarcosine, α -aminocaprylic acid, and 5-hydroxypipericolic acid, but is identical with none of these, as was proved by

Table 1. *Oenanthe aquatica* cultivated in 250 ml. normal (A) and glycine-containing (B) media. Yield of fresh tissue, "carbohydrate", "protein", and the nitrogen fractions soluble and insoluble in cold 8 % TCA. "Protein" calculated from the insoluble N, assuming a protein N content of 15 %.

Culture medium 250 ml.	Total fresh material g.	Insoluble dry material			N		Soluble N	
		Carbo- hydrate g	Protein g.	Total g.	mg.		mg.	% of total N
A Normal.....	17.5	0.79	0.22	1.01	33.0		1.47	4.3
B Glycine.....	19.5	0.58	0.23	0.81	34.4		19.50	36.2

mixed runs with authentic compounds. The neomorph also contains relatively more β -alanine (No. 32) and less free threonine (No. 9) and aspartic acid (No. 16) than the normal plant. A basic peptide G, present in the normal plant, seems to be absent from the neomorph.

These results were confirmed by paper chromatography of 70 % alcohol extracts from other samples of the normal plant and the neomorph.

Discussion

It has been shown by earlier workers (Virtanen and Linkola 1946, 1957; Steinberg 1952) that addition of amino acids to the nutrient solution supplied to higher plants may produce morphological changes, called "frenching" by Steinberg (*loc. cit.*). In the present case the culture experiments show that an excess of glycine in the nutrient solution is in the long run toxic to normal seedlings of *Oenanthe aquatica* completely preventing their growth, but nevertheless allowing them to develop a new but simple form which is able to grow and proliferate in the same solutions. It thus seems that it is differentiation rather than cell division, which is inhibited by glycine.

The results of chemical analyses are in agreement with the view that the partial inhibition is due to a competitive antagonism between glycine and one or several other amino acids, the sequence of some enzymic reactions being blocked by glycine. Owing to this, the normal course of synthesis is upset and the cellular constituents are formed in abnormal proportions. The accumulation of soluble amino acids in the neomorph indicates that protein synthesis is disturbed, and this may result in a deficiency in the enzyme system. The unknown substances A and B, found in the chromatogram, may represent precursors in the blocked metabolic system, which are enriched in the neomorph. On the other hand, the carbohydrate metabolism is affected too, since in normal plants both soluble and insoluble carbohydrates are definitely

higher than in the neomorph. In conformity with this fact, the mechanical tissues seem to be lacking, and the vascular strands are poorly developed in the neomorph. Moreover, the parenchymatous tissues seem to be extraordinarily rich in pectic substances, to judge from their staining with ruthenium red. On the whole, the neomorph exhibits a profound physiological and morphological adaptation to glycine and submerged life at the same time, their genotype, however, not being irreversibly changed.

Summary

A profound morphological and physiological change takes place in *Oenanthe aquatica* during growth in a nutrient solution containing sucrose and glycine, whereas normal plants are produced if glycine is omitted. A comparative chromatographic study shows that the neomorph contains the same free sugars, glucose and fructose, in lower concentrations, and the same free amino acids in about 10 times higher concentrations than the normal plant. In addition it contains two unknown ninhydrine-positive compounds not present in the normal plant. One of these is probably an amino acid hitherto not found in higher plants, the other one a peptide.

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Studies on the Relation between Auxin Activity and Chemical Structure II.

Optically Active 1,2-Dihydronaphthoic Acids-(1) and the Hydrogenated Derivatives of Naphthoic Acid-(2)

By

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(Received September 2, 1957)

Veldstra (19), Mitsui (12) and Wain (21) have independently found a significant difference in the physiological activity between the optical antipodes of hydrogenated naphthoic acids-(1). The present author (4) has also studied the optical isomers of 1,4-dihydro- and 1,2,3,4-tetrahydronaphthoic acid-(1) for their activity in the *Avena* curvature test, in the pea test, in the root initiation, etc. The results agree with those of the above-mentioned authors.

Reported here are experiments using D-, L- and DL-1,2-dihydronaphthoic acid-(1), as well as naphthoic acid-(2) and its derivatives. They present some facts which will contribute to the problem of the relation between molecular structure and auxin activity, since surface activities and molecular configurations of naphthoic acid derivatives have been studied by Mitsui and his collaborators (13, 14).

Methods

Methods were essentially the same as in the previous paper (4), except the straight growth test. For this test, sections were cut off from the third internode of etiolated pea seedling which had been cultured in the darkroom at 25°C for 7 days. The internode was about 15—20 mm. long, and the section was 5.25 ± 0.017 mm. in the average length. Sections were floated in test solutions for 24 hours, and then their length was measured again. The measurement was made with a stereoscopic microscope, placing the section on a rule of 0.01 mm. scale.

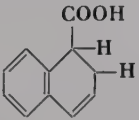
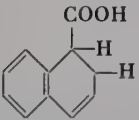
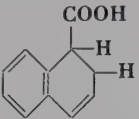
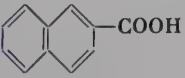
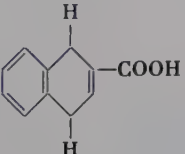
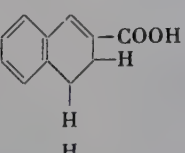
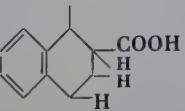
Those compounds were described as inactive that did not cause positive reaction by the solution of 300 mg./l. in the pea test, and by the 1 per cent lanolin paste in the callus formation and the bud inhibition.

The abbreviations, NcA-(1), NcA-(2) and IAA, stand for naphthoic acid-(1), naphthoic acid-(2) and indole-3-acetic acid, respectively.

Results

Pea test. The activity of D- and DL-1,2-dihydro-NcA-(1), as shown in Table 1, was about the same as that of D-1,4-dihydro-NcA-(1) and IAA (4).

Table 1. Activity of naphthoic acid derivatives in the pea test.

Compound	Formula	Effective conc. range, mg./l.	Relative activity ¹
D-1,2-Dihydro-NcA-(1)		0.5—300	100
L-1,2-Dihydro-NcA-(1)		Inactive	0
DL-1,2-Dihydro-NcA-(1)		0.5—300	100
NcA-(2)		Inactive	0
1,4-Dihydro-NcA-(2)		Inactive	0
3,4--Dihydro-NcA-(2)		Inactive	0
DL-1,2,3,4-Tetrahydro-NcA-(2)		Inactive	0

¹ $(5.7 \times 10^{-5} \text{ divided by molar concentration of the compound necessary to induce the same reaction as } 5.7 \times 10^{-5} \text{ M IAA}) \times 100.$

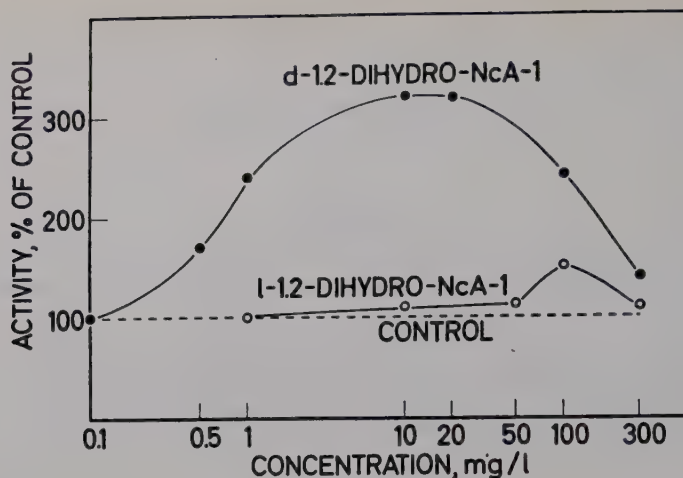


Figure 1. Effect of D- and L-1,2-dihydronaphthoic acid- (1) on the straight growth of pea stem section. Each point represents the average of 10 pieces. Differences among 1, 10, 50 and 300 mg./l. of L-isomer and control, respectively, are not significant at 1 per cent level.

However, the inward curvature of split sections was not caused by the L-isomer at any concentration up to 300 mg./l., above which the material became flaccid owing to injury. The derivatives of NcA-(2) tested were also inactive.

Pea straight growth test. The effect of D- and L-1,2-dihydro-NcA-(1) on the straight growth of pea stem section is shown in Figure 1. The D-isomer remarkably promoted the growth, and its effective concentration range was nearly equal to that observed in the pea curvature test. On the other hand,

Table 2. Effect of L-1,2-dihydronaphthoic acid-(1) on the growth effect of the D-isomer, as determined by the straight growth of pea stem section. Average of three sets of experiment.

Concentration mg./l. L-isomer	Concentration mg./l. of D-isomer		
	0	1	100
0	100	124 ²	132 ³
1	101	118 ²	130 ³
10	100	119 ²	131 ³
50	101	118 ²	131 ³
100	113 ¹	118 ²	125 ³

¹ Difference from control significant at 1 per cent level.

² Differences within this series are not significant at 1 per cent level.

³ Differences within this series are not significant at 1 per cent level.

Table 3. *Effective concentration range and relative activity of naphthoic acid derivatives on the callus formation.*

Compound	Effective conc. range %	Relative activity ¹
D-1,2-Dihydro-NcA-(1)	0.01—1	> 100
L-1,2-Dihydro-NcA-(1)	Inactive	0
DL-1,2-Dihydro-NcA-(1)	0.01—1	> 100
NcA-(2)	Inactive	0
DL-1,2-Dihydro-NcA-(2)	Inactive	0
1,4-Dihydro-NcA-(2)	Inactive	0
3,4-Dihydro-NcA-(2)	Inactive	0
DL-1,2,3,4-Tetrahydro-NcA-(2)	Inactive	0

¹ (0.1 divided by the percentage concentration of the compound necessary to induce the same reaction as 0.1 per cent IAA) $\times 100$.

the L-isomer slightly promoted the growth at a comparatively high concentration.

Interaction between D- and L-1,2-dihydro-NcA-(1). Wain *et al.* (21) have shown, using phenoxy- and naphthoxy-propionic acid derivatives in the *Avena* cylinder test, that the activity of D-isomers was reduced by the addition of the respective L-isomers. Therefore, the antagonistic action of L-1,2-dihydro-NcA-(1) against its D-isomer, was tested for the straight growth of pea stem section. The final lengths of stem sections expressed as percentage of control are represented in Table 2. No competitive antagonism was observed between the D- and L-isomers even for a wide range of concentration. Although the L-isomer was slightly active at 100 mg./l., no additive, nor antagonistic, effect to the D-isomer was observed.

Callus formation. When lanolin paste was applied on the cut surface of etiolated epicotyle of *Vicia Faba*, D- and DL-1,2-dihydro-NcA-(1) were active

Figure 2. *Callus formation observed 7 days after treatment at the apical cut surface of epicotyl segment with 0.1 per cent lanolin paste. (A) Control; (B) Indole-3-acetic acid; (C) D-1,2-Dihydronaphthoic acid-(1); (D) L-1,2-Dihydronaphthoic acid-(1); (E) DL-1,2-Dihydronaphthoic acid-(1).*

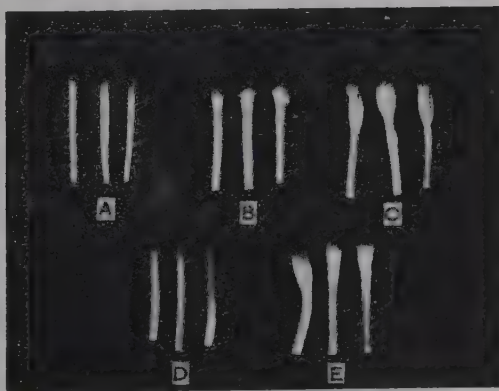


Table 4. *Effect of 1 per cent lanolin paste of optically active 1,2-dihydronaphthoic acid-(1) on the growth of lateral bud of etiolated pea seedling. Each value represents the average of 15—18 samples.*

Compound	Length of lateral bud after 8 days (% for control)	Inhibition %
D-1,2-Dihydro-NcA-(1)	63 ¹	37
L-1,2-Dihydro-NcA-(1)	94	6
DL-1,2-Dihydro-NcA-(1)	62 ¹	38
Control (pure lanolin)	100	0
IAA	0	100

¹ Significantly different from control at 1 per cent level.

in the callus formation, whereas the L-isomer and five derivatives of NcA-(2) were inactive (Table 3). The former two substances induced callus even at 0.01 per cent as lanolin paste. At 1 per cent, the treated part of the material turned dark brown, the zone just below it swelling later. The effect of 0.1 per cent paste is shown in Figure 2. The callus-forming activity of D- and DL-1,2-dihydro-NcA-(1) was higher than that of IAA and D-1,4-dihydro-NcA-(1), and nearly corresponded to that of L-1,2,3,4-tetrahydro-NcA-(1).

Bud inhibiting effect. Etiolated pea stem was decapitated just above the first lateral bud, and the cut surface was smeared with 1 per cent lanolin paste of compounds to be tested. The growth of the lateral bud to follow was measured. As shown in Table 4, D- and DL-dihydro-NcA-(1) slightly inhibited the elongation of the lateral bud, but their inhibiting effect was far weaker than IAA. L-1,2-Dihydro-NcA-(1) showed no significant effect.

Discussion

Generally speaking, D- and DL-1,2-dihydro-NcA-(1) cause strong auxin effects. They are as effective as, and even more effective than IAA, in the pea test and in the callus formation, although less effective in the bud inhibition. The L-isomer, on the other hand, was not found active in the three tests, except that it slightly promoted the growth of pea stem section at a very high concentration. The derivatives of NcA-(2) were also inactive in the tests so far tried.

Using about twenty-five compounds, Paleg and Muir (18) found no parallelism between the surface activity and the auxin activity. According to Mitsui and Fujita (14), the half suppression values of derivatives of NcA-(1)

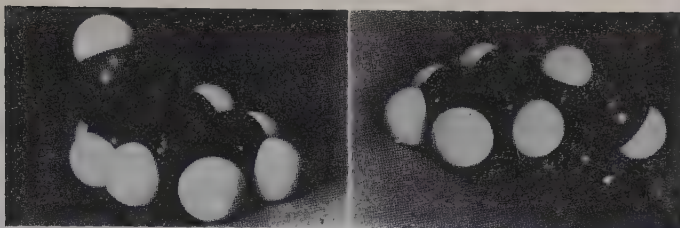


Figure 3. One pair of model configuration of one of optical isomers of 1,2-dihydronaphthoic acid-(1) by Mitsui (15).

and NcA-(2) did not differ considerably from one another. But there are remarkable differences in the auxin activity among them.

According to Mitsui (15), one of the optical isomers of 1,2-dihydro-compound may take either of the two stereo-configurations, as shown in Figure 3, namely, the one having the carboxyl group at the polar position against the ring system and the other at the equator position. This is because the 1,2-dicalin-ring of this compound is always undergoing inversion. It is not determined to which of the isomers, D and L, the pair of configurations in Figure 3 corresponds. However, one of the isomers should have the configurations which are mirror images of the other. The probability of an isomer having the carboxyl group at the polar position should be equal to that of the other isomer. Hence the difference in the auxin activity between D- and L-1,2-dihydro-NcA-(1) cannot be explained by Veldstra's requirement for the polar position of carboxyl group (20).

Inaba, Koshimizu and Mitsui (3) have reported that D- and L-1,2-dihydro-NcA-(1) are obtained by dehalogenation, respectively, of L- and D-1,2,3,4-tetrahydro-3,4-dibromo-NcA-(1), which are derived by bromination of L- and D-1,2,3,4-tetrahydro-NcA-(1), respectively. By hydrogenating the dihydro-acid, Mitsui (13) showed that D- and L-1,4-dihydro-NcA-(1) have the same configuration as L- and D-1,2,3,4-tetrahydro-NcA-(1), respectively. Hence D-1,4-dihydro-, L-1,2,3,4-tetrahydro- and D-1,2-dihydro-acids are suggested to belong to the same absolute optical configuration, and L-1,4-dihydro-, D-1,2,3,4-tetrahydro- and L-1,2-dihydro-acids to the other. Matell (5, 6, 7, 8, 9, 10) examined the auxin activity of several α -aryloxy-alkylcarboxylic acids, and concluded that members belonging to D-series are active, while those of L-series are inactive or even anti-auxins. So if his expectation is applied here, the active isomers, namely, D-1,4-dihydro-, L-1,2,3,4-tetrahydro- and D-1,2-dihydro-acid may belong to D-series, and the inactive ones, L-1,4-dihydro-, D-1,2,3,4-tetrahydro- and L-1,2-dihydro-acid, to L-series. That remains to be proved.

Hansch and Muir (2) advanced the two-point-attachment theory which requires at least one of the two ortho positions of the ring system to be free. Some evidences support the theory (*e.g.* McRae and Bonner, 11, Overbeek *et al.*, 17), but some others do not (*e.g.* Osborne *et al.*, 16). Since the hydrogen atom of D-1,2-dihydro-NcA-(1) may easily be replaced, the activity of this substance can be explained with the two-point-attachment theory. However, NcA-(2) derivatives and L-1,2-dihydro-NcA-(1) are inactive, in spite of that their position, correspond to the ortho-position of the phenoxy-compounds, is free just as the active derivatives of NcA. A theory is needed which can explain these case.

Just as pointed out in the previous paper (4), Went's requirement (22) of the existence of at least one carbon atom between the ring system and the carboxyl group, is to be modified for D- and DL-1,2-dihydro-NcA-(1). Further discussions will appear elsewhere.

Summary

Using the etiolated pea seedling or the etiolated epicotyl of *Vicia Faba*, D- and DL-1,2-dihydronaphthoic acid-(1) were shown to be active in the curving of split stem, in the straight growth of excised stem piece, in the callus formation and in the bud inhibition. The L-isomer of this acid was inactive in all of these tests. The derivatives of naphthoic acid-(2) so far tested were inactive in the pea test and in the callus formation. Hence Went's and Veldstra's structural requirements were not sufficient.

There was no competitive antagonism between the D- and L-isomers of 1,2-dihydronaphthoic acid-(1).

The author wishes to thank Professor Joji Ashida for his guidance, Mr. Makoto Inaba for the compounds on which the present research depends, and Professor Tetsuo Mitsui for giving advices on chemical problems.

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The Activity of S-(Carboxymethyl)-Dimethyldithiocarbamate as an Auxin

By

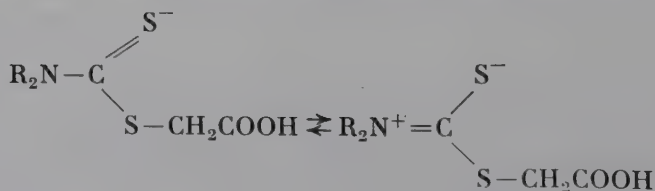
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The growth regulating properties of S-(carboxymethyl)-dimethyldithiocarbamate (G-33) have been recently described (van der Kerk *et al.*, 1955; van Raalte *et al.*, 1955). This compound, as well as several other structurally related thiocarbamates, was found to be active in causing leaf epinasty, leaf deformation, and root initiation. All were active as auxins, though the activity of G-33 in the pea, Avena cylinder, and sunflower stem tests was stated to be, respectively, 1, 5, and 5 per cent that of indoleacetic acid (IAA) (van der Kerk, 1955; see also Veldstra, 1956). As with other growth substances, alkyl replacement of both hydrogens on the carbon adjacent to the carboxyl group results in loss of biological activity.

If G-33 is an auxin, it is unusual in that it does not possess the unsaturated ring plane characteristic of the auxins. Considering this structural requirement, van der Kerk *et al.* (1955) suggested that internal electron shift occurs in the active thiocarbamates to yield an unsaturated pseudo-ring system having the required groups and spatial configuration for auxin activity:



It occurred to us that the auxin activity of G-33 might not be related casually to pseudo-ring formation, but rather might result either from the "acid effect" (Bonner, 1934; Thimann, 1935) or from auxin-sparing action (Skoog *et al.*, 1942), particularly since relatively high concentrations are required for activity. These alternative explanations to the stereochemical interpretation of van der Kerk *et al.* are examined in the present paper.

Methods

The Avena cylinder test was used in the experiments described. Three-millimeter sections were cut by a van der Weij guillotine 3 mm. from the tip of 70-hour etiolated coleoptiles; the foliage leaf was not removed. Sections were randomized for use in the tests. For each treatment 10 sections were floated on 2 ml. of the test solution in a 5 cm. petri dish and kept in the dark at 25°C. All test solutions contained 2 per cent sucrose. The lengths of the sections were measured 20 hours later by means of a dissecting microscope with an ocular micrometer. All operations involving tissues, except extraction, were carried out in a standard auxin bioassay room maintained at 25°C and 92 per cent relative humidity, under safelights excluding light with wavelength less than 540 mμ.

The IAA labeled at the methylene carbon with C¹⁴ (Stutz *et al.*, 1951) was purified by chromatography (Gordon and Paleg, 1957) before use. Its specific activity was 1.35×10^5 disintegrations/min.μg (10.6 mc/mM). The beta activity of the samples was measured with a Packard windowless flow counter adapted for the proportional region. All counts were corrected for background and, where pertinent, for sample absorption.

The effect of G-33 on the release of bound C¹⁴-IAA was determined as follows. A group of 250 5-mm. coleoptile sections, cut 3 mm. from the tip,* was immersed for 15 minutes in 5 ml. of water containing 15.2 μg C¹⁴-IAA per ml. The sections were caught on a strainer and washed with distilled water. They were then permitted to grow for 20 hours on 6 ml. 0.005 *M* maleate buffer, pH 5.0, in the dark at 25°C. During this period the sections almost doubled in length. They were then washed again with distilled water, blotted and divided into two lots of 125 sections each. One lot was replaced in 6 ml. of fresh sucrose-maleate buffer solution, the other in buffer containing 100 μg G-33 per ml. At 1, 2, 4, 6, and 8 hours afterward, 25 sections and 1 ml of medium was removed from each lot and extracted twice with either at 0—4°C, each extraction lasting one half hour. The pooled extracts were evaporated and taken up in 60 λ MeOH (purified by distillation from Zn-KOH) that contained 20 μg IAA. A 50 λ aliquot of each sample was resolved by chromatography as described elsewhere (Gordon and Paleg, 1957). After drying of the chromatogram, the IAA spot was delineated under ultraviolet light and cut out. The remainder of the strip was then segmented. Each segment was eluted with MeOH and the eluates were deposited on planchets for counting.

Results and Discussion

Acid effect. — The growth increments occurring at various concentrations of G-33 and IAA without added buffer are given in Figure 1A. It required

10/ μ g G-33 per ml. to cause the same increment in growth, 1 mm, that was caused by 0.1 μ g IAA per ml. The activity of the carbamate is thus about 1 per cent that of IAA. No growth acceleration was detected in G-33 solutions at concentrations at or below 3 μ g/ml. At a concentration of the carbamate of 10 μ g/ml, the pH was 4.8, while at 50 and 100 μ g/ml the pH was, respectively, 3.8 and 3.6. Though a greater growth increment would be anticipated at these lower pH values if we were dealing with an acid effect (Bonner, 1934), Figure 1A shows that less, not more, growth took place. Furthermore, Figure 1B shows the growth increments that resulted when all test solutions were buffered to pH 5.0 in 5×10^{-3} M maleate. It is apparent that G-33 still causes the same maximum growth stimulation, though the inhibition at high concentrations of the carbamate indicated in Figure 1A has been shifted to the right in the buffered system. It may be concluded from these results that the activity of G-33 does not derive from the acid effect.

Growth stimulation followed by growth inhibition is a typical response to increasing concentrations of auxin. This phenomenon is also observed with G-33 (Figure 1). When curve A is compared with curves B and C, it is apparent that a tenfold higher concentration of G-33 is required for inhibition in the buffered systems. Thus the inhibition at high concentrations of G-33 is counteracted either by the presence of the buffer ions or the lowered H^+ concentration. It may also be observed that the maximum growth increment obtained with G-33 is about half that obtained with IAA, and that G-33 in the buffered systems at pH 5 has a tendency to inhibit growth slightly at low concentrations.

Auxin sparing. — Auxin sparing action is a rather loose term that may include several phenomena that increase the amount of IAA available to physiologically active sites. First, a substance may preferentially compete with endogenous IAA for binding loci that are not physiologically active. Second, it may liberate IAA already bound at inactive sites. Third, it may inhibit catabolism of endogenous IAA.

Lowering the endogenous auxin level of the section should permit a test of the first and third of the above possibilities. Rietsma (1949) has shown that soaking of coleoptile sections before adding IAA enhances markedly the sensitivity of the sections to the IAA. Presumably this results, at least in part, from depletion of the endogenous auxin in tissue unable to synthesize auxin. (It has its analogy in the first decapitation of coleoptiles in the standard *Avena* curvature test as a means of depleting auxin in subterminal tissues (Leopold, 1955).) Therefore, sections were soaked in distilled water for 5 hours, blotted, and placed in buffered solutions with different concentrations of G-33 and IAA. The growth curves are shown in Figure 1C. They indicate that presoaking of the section did not lower activity of G-33. On the contrary,

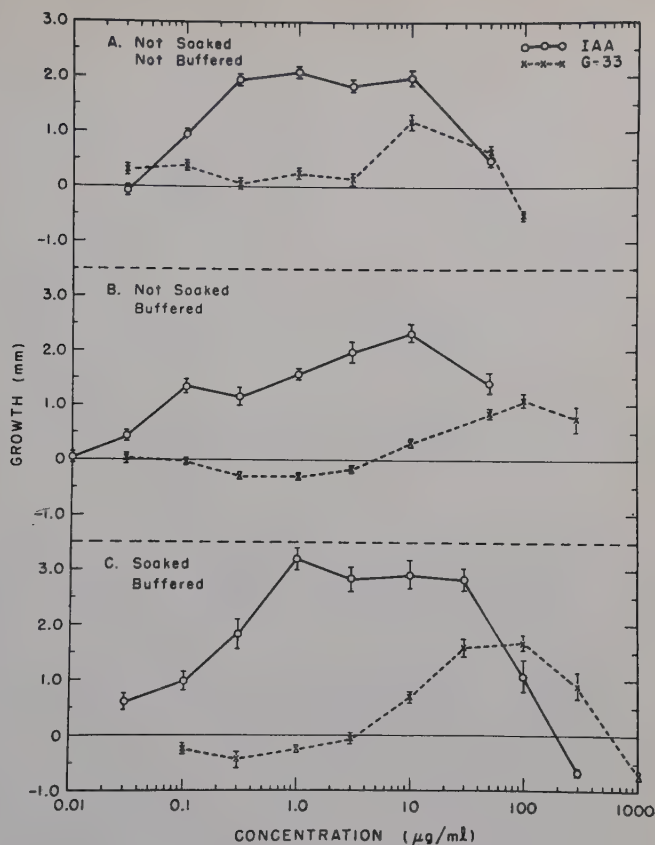


Figure 1. Growth responses of coleoptile sections in various concentrations of G-33 and IAA. The growth increments plotted represent the increase in length of sections over the length in control solutions without added IAA or G-33. The bracket denotes the standard error of each increment.

the activity of G-33 increased over twofold, while the activity of IAA increased about $1\frac{1}{2}$ times. Hence, it is unlikely that the activity of G-33 is caused by competition with endogenous IAA for inactive sites. Similarly, the activity of the carbamate cannot be attributed to inhibition of a catabolic system responsible for depletion of endogenous IAA.

The above conclusions rest on the assumption that pre-soaking does in fact lower the auxin content of the coleoptile sections. This was verified by direct determination of the free auxin in pre-soaked sections. Half of a group of 1650 3-mm. sections was dropped into ether at -20°C and extracted for one half hour at 0°C . The ether was decanted and the extraction was repeated three times. The other half of the group was soaked in distilled water for 5 hours, blotted, and then extracted with ether in the same manner. The pooled ether extract from each treatment was reduced in volume and evaporated on an $8\times 11\times 1.5$ mm. block of 1.25 per cent buffered agar (Larsen, 1955), the evaporation being facilitated by a stream of water-scrubbed nitrogen. After a diffusion period of about 2 hours in a water-saturated atmosphere, the agar blocks were cut into 12 equal sub-blocks and assayed by the standard *Avena* curvature method. Those tissues that were not pre-soaked assayed 3.8 ± 0.44

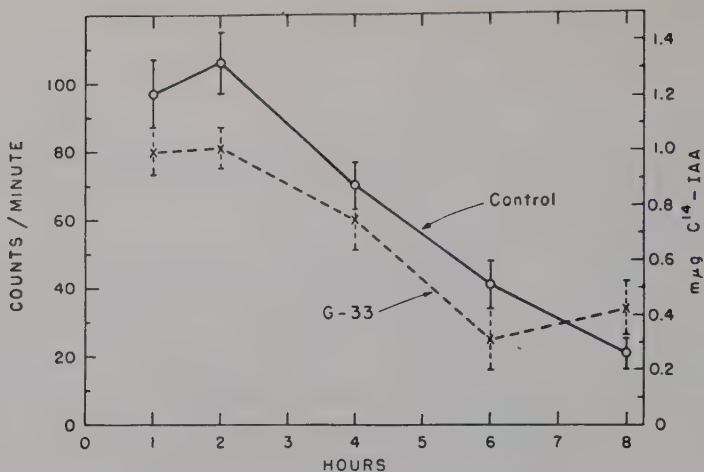


Figure 2. The effect of G-33 on the amount of free IAA obtained from coleoptile sections previously exposed to C^{14} -labeled IAA. Each point represents the IAA extracted from 25 sections and 1 ml of the medium at various times after immersion of 125 sections in 6 ml of sucrose buffer with and without G-33.

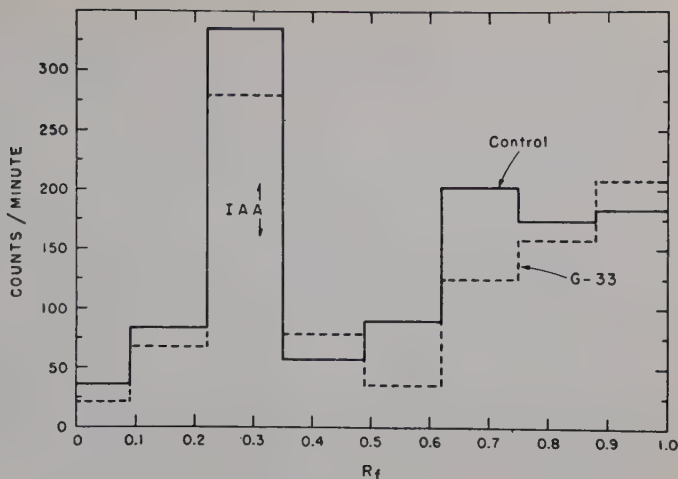
$\times 10^{-6}$ μ g, per section, whereas the pre-soaked tissues assayed $2.0 \pm 0.36 \times 10^{-6}$ μ g, per section. Soaking, therefore, did reduce the free auxin content by about 50 per cent.

It is probable that IAA in tissues is in part rendered unavailable for auxin or hormonal function by binding at loci other than those connected with the auxin action mechanism (Gordon, 1954). By binding we mean an immobilization of auxin in any complex wherein the structural integrity of the auxin is either retained or is reversibly reattainable. The second of the possible mechanisms suggested above for auxin sparing action is that the G-33 causes the release of bound IAA, raising the auxin level and thereby increasing growth. This possibility was tested by allowing coleoptile tissues to bind C^{14} -IAA and then determining whether G-33 would affect the level of ether-extractable and water-diffusible labeled IAA (see Methods).

Figure 2 shows the amounts of C^{14} -IAA, as isolated by chromatography, that were extracted at various time intervals from coleoptile sections and media with and without G-33. It is evident that exposure of the tissues to G-33 does not result in an accelerated release of the C^{14} -IAA that had been taken up by the tissues between 20 and 28 hours before. If anything, the amounts of C^{14} -IAA obtained were slightly less in the presence of the carbamate. If the binding of C^{14} -IAA is considered indicative of the binding of endogenous auxin, this experiment indicates that G-33 does not cause the release of bound auxin.

The same conclusion can be drawn from Figure 3, which shows the C^{14}

Figure 3. The distribution of C^{14} activity in chromatograms of the ether extracts of coleoptile sections exposed to C^{14} -labeled IAA and subsequently to G-33.



activity at each R_f segment of the chromatographed extracts. Each datum in the figure represents the sum of the activities at 1, 2, 4, 6, and 8 hours, the individual chromatograms being qualitatively similar. Exposure of the tissues to G-33 not only resulted in no increased liberation of C^{14} -IAA, but also had no significant effect on the liberated amount of radioactive, ether-soluble, decomposition product that was produced from C^{14} -IAA. From the distribution of activity in the frontal region of the chromatogram, it may be inferred that the product is less ionic than IAA.

Heath and Clark (1956) have shown that a number of chelating agents, including diethyldithiocarbamate, have auxin activity upon sections of the wheat coleoptile. They discussed the possibility that the mechanism of auxin action primarily involves the formation of metallo-organic complexes (*cf.* also Bennet-Clark, 1956). It is possible that the mode of action of G-33 falls into the same category as the action of the recognized chelating compounds. If so, the deductions here made that neither the acid effect nor auxin sparing action causes the auxin activity of G-33 may apply also to the rather diverse molecular structures examined by Heath and Clark. It may also be pointed out that the observations in the present paper do not clarify the possibility suggested by van der Kerk *et al.* that a pseudo-ring stabilization of the G-33 is involved in its action as an auxin.

Summary

The possibility that the activity of S-(carboxymethyl)-dimethyldithiocarbamate (G-33) results from an 'acid effect' or from auxin-sparing action was

examined experimentally. Response to this carbamate in the *Avena* section test is not directly related to the pH of unbuffered test systems, nor is the activity changed significantly in a medium buffered to a constant pH. Activity of G-33 does not decrease but actually increases when tested on sections depleted of their endogenous auxin. Furthermore, G-33 causes no liberation of bound C^{14} -indoleacetic acid from sections previously exposed to the labelled auxin. It is concluded that the activity of G-33 does not derive from the enhancement of tissue response to endogenous auxin. These observations permit the less equivocal characterization of G-33 as an auxin that does not possess the closed ring system usually associated with auxin activity.

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Tuberization in the Potato Plant

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The precise stimulus resulting in tuberization of the potato plant (*Solanum tuberosum* L.) is still unknown. Several possible explanations for this response have been presented from time to time; the oldest perhaps being that of Bernard (1902). He gave evidence for the association of the cells of the stolon tips with a fungus which he believed to be responsible for tuber formation. Certain persons still subscribe to this explanation (Magrou, 1938). Werner (1934, 1940) bases an explanation of his results with tuberization on carbohydrate-nitrogen relationships. Factors resulting in a shift of the ratio toward a surplus of carbohydrates as short daylengths, low temperatures, or low nitrogen supply favoured tuber formation. Later Driver and Hawkes (1943) demonstrated that many potato varieties form tubers early when subjected to short days. Gregory (1956) postulated the formation of a tuber forming stimulus with short day conditions (8 hr.) and low night temperatures (below 20°C.), this stimulus moving to the stolon tips and resulting in tuber formation. Gregory's experiments were carried out in the phytotron at California Institute of Technology where he undoubtedly had excellent control of environmental conditions.

Recently several workers (Barker, 1953; Mes and Menge, 1954; Chapman, 1955) have reported methods of culturing nodes from potato plants under aseptic conditions on nutrient media. These techniques provide a convenient method for determining the presence or absence of the tuber-forming stimulus at any location in the potato plant.

Two distinct processes, tuber induction and tuber enlargement, should

be recognized, tuber enlargement being the first visible evidence of tuber induction. The object of this research was to determine the plant parts in which the stimulus is formed and to gain additional information about factors affecting its movement and expression in tuber formation.

Materials and Methods

The experimental potato plants of Triumph variety were grown in the greenhouse at Lincoln, Nebraska. Seed pieces were planted in either sand or vermiculite and held at 21°C. with 18-hr. daylength until the sprouts were 5–10 cm. long at which time they were transplanted into the nutrient tanks. The seed piece with the attached sprout and roots was placed on an inverted pot just above the level of the liquid nutrient solution. Development of roots, stolons and tubers could be easily observed at any time by raising the tar paper tank cover. The nutrient tanks were 2×2 m. and 18 cm. deep; 48 plants were grown in each tank. The nutrient solution was maintained at a depth of about 6–8 cm. and was renewed at weekly intervals.

Aeration was provided from a utility supply line attached to porous carbon cylinders at nine locations in each tank. The nutrient solution has been used for growing potatoes in gravel at this laboratory for many years (Honma, 1956). The solution was supplemented with 2 g. of Sequestrene NaFe per 100 gal. Excellent growth of plants was always obtained. Tubers enlarged above or submerged in the medium depending on the length of the stolon. Air temperature surrounding the plants averaged near 20°C.

Three contrasting photoperiod treatments were utilized: (1) a 9-hr. daylength (natural light) followed by a 15-hr. dark period; (2) an 18-hr. daylength followed by a 6-hr. dark period and (3) continuous light. For the long photoperiods, natural daylight was extended with Mazda bulbs which provided 100–500 f.c. light intensity. Plants grown with a 9-hr. photoperiod are sometimes referred to as induced plants, while those grown with 18- or 24-hr. photoperiods are referred to as non-induced plants.

In the grafting experiments leafy tops, 30–60 cm. in length, from induced plants were cleft grafted to stocks of both induced and non-induced plants. Similarly tops from non-induced plants were grafted to stocks of both types of plants. Tuber formation and other growth manifestations were then carefully observed.

To determine the status of the tuber-inducing stimulus in the plants, cuttings taken from the upper one-third, center one-third, and basal portions of stems were cultured in two different ways: (1) After removing the leaves, cuttings were planted in moist sand in a cool (20°) rooting bench; (2) After removing the leaves, cuttings were surface sterilized (Chapman, 1955) following which individual nodes including 2–6 mm. of the stem were excised and transferred to sterile media. The medium contained White's (1943) mineral nutrient solution supplemented with 2 per cent sucrose and 1 per cent agar. Fifty ml. of the medium was poured into individual 125-ml. Erlenmeyer flasks used as culture containers. They were autoclaved for 20 min. at 15 lb. pressure and allowed to cool and solidify before use. The cuttings in the sand filled bench and nodes in aseptic cultures were examined at intervals for the presence of tubers.

Experimental Results

Daylength effects on tuberization and plant growth. — Typical short-day plants formed a relatively small number of tubers on short stolons (Figure 2). At the time of tuber formation or shortly thereafter vegetative development of both the tops and roots ceased. The growing points were inactive with few or no young expanding leaves. The root tips stopped elongating and soon lost their pearly white color. Long-day (18 hr. followed by 6 hr. darkness, or continuous light) plants, however, held under similar conditions except for daylength, developed extremely vegetative tops with active growing points and very long (frequently 60 cm. or more) branched stolon systems (Figure 2). The root system was extensive and continuous in its development. Tubers formed in large numbers 3—5 weeks after the short day plants had tuberized (Table 1).

Location of stimulus formation. — To determine more precisely the organs or parts responsible for the photoperiod response on tuberization, plants were treated differentially as described in Table 1. When the terminal leaf cluster¹

Table 1. *Influence of daylength on tuberization and growth.*

Treatment ¹	Plants tuberized in the specified number of days (12 plants per treatment) ²							No. of Tubers	Mean fresh weight per plant (g.) of various plant parts			
	26	30	33	36	40	44	48		Tubers	Tops	•Under ground stems and stolons	Roots
9 W	4	12	12	12	12	12	12	27	84	105	9	41
18 W	0	0	0	0	0	2	5	56	16	471	41	148
18 T } 9 B }	0	0	0	0	0	2	7	48	20	282	29	95
9 T } 18 B }	1	2	10	12	12	12	12	37	65	154	17	72

¹ 9 W — 9-hour daylength (whole plant).

18 W — 18-hour daylength (whole plant).

18 T 9 B — 18-hour daylength (terminal leaf cluster) 9-hour daylength (basal leaves).

9 T 18 B — 9-hour daylength (terminal leaf cluster) 18-Hour daylength (basal leaves).

² Short-day treatments were applied from October 12 to November 13, at which time the plants were returned to 18-hr. photoperiods. Days are counted from the date that the short-day treatments were started.

¹ The terminal leaf cluster included the growing point and young rapidly expanding leaves usually less than 5 cm. in length from the main stem to the tip of the terminal leaflet.

Table 2. *Influence of number of successive short-day cycles on tuberization.*

Treatment	Plants tuberized 22 days after starting short photoperiods (8 plants per treatment)
1—24 hours, continuous light	0
2— 7 cycles, 9-hour daylength ¹	1
3—14 cycles, 9-hour daylength ¹	8
4—21 cycles, 9-hour daylength ¹	8

¹ Short day treatments started October 18 when the plants were 35-cm. tall.

only received the short photoperiod (9T 18B), tuberization occurred at the same time as the short-day check plants (9W). Similarly when the terminal leaf cluster received an 18 hr. photoperiod (18T 9B) tuberization occurred at about the same time as the long-day check plants (18W).

Number of short-day cycles. — Potato plants subjected to only 7 short-day cycles or less failed to tuberize consistently (Table 2), while 14 or more short-day cycles before returning the plants to a continuous light regime, invariably resulted in tuber formation. Four weeks following tuberization, tubers on plants that had received only 14 short-day cycles were developing stolons from the eyes (Figure 1). As reported below, a similar response was also observed when long-day scions were grafted to tuberized stocks.

Differential daylength treatment of divided plant tops. — In this experiment the terminal growing point of sprouts about 7 cm. long was removed, forcing lateral bud growth. The plants were maintained under long-day conditions for 14 additional days after which each plant was pruned to two vigorous shoots and differential treatments were applied. One shoot of each plant was given a 9-hr. daylength while the other received an 18-hr. day. Twenty-four cycles were given and then all plants were returned to a daylength of 18 hr. We expected, of course, to obtain tubers on these plants at about the same time as the short-day controls. The results in this respect are given in Table 3. More surprising, however, was the unilateral development of tubers, the tuber-forming stimulus seemingly being confined to one-half of the stem on the short-day side of the plant (Fig. 2). Lateral diffusion around the stem to the long vegetative stolons on the long day side occurred only very slowly. This response was observed on each of 24 plants included in this treatment. Essentially, the two halves of the stem base appeared to act as separate entities.

Graft transmission of the tuber forming stimulus. — Long-day stocks grafted to an induced (short-day) scion, set tubers within 14 days. The grafting of long-day scions to long-day stocks did not result in tuber formation. After the grafts were made, all plants were held under 18-hr. day, non-inducing

Figure 1. *Effect of duration of daylength treatment on tuberization.* Plants at the left received 14 short days (9 hr.). The plant at the right received 21 short days (9 hr.). Note tubers from plants which received only 14 short days developed sprouts from the eyes.



Figure 2. *Effect of daylength on potato tuberization.* Plant at left and left one-half of center plant was given 24 short-day (9-hr) cycles. Plant at the right and right one-half of center plant was given 18-hr. days continuously. All plants grown in liquid nutrient solution in a common tank in the greenhouse.



conditions. Of particular interest was the response of short-day tuberized stocks when long-day scions were attached. Several such stocks again started to grow vegetatively forming stolons from the eyes of tubers.

Effect of pruning on plant development. — Following the lead that the terminal growing points of the stems are the photoperiodically sensitive regions that control development of the tuber-inducing stimulus, potato plants

Table 3. *Effect of differential daylength treatments on tuberization.*

Treatment	Number of plants per treatment	Plants tuberized in the specified number of days ³							
		25	28	31	33	38	43	53	63
Whole plant 18 hr.	12	0	0	0	0	0	0	5	12
One shoot 9 hr. ¹ }	24	0	8	24	24	24	24	24	24
One shoot 18 hr. }									
Whole plant 9 hr. ²	12	7	12	12	12	12	12	12	12

¹ Plants were treated differentially; one shoot received 9-hr. days from October 1 to October 24, the other shoot received 18-hr. days continuously.

² Whole plants received 9-hr. daylengths from October 1 to October 24

³ Number of days from the start of short-day treatment (October 1).

Table 4. *Influence of several plant pruning procedures on tuberization.*

Treatment ¹	No. of Plants per Treatment	Plants tuberized in the specified number of days ²									
		26	30	33	37	41	48	58	69	78	
9 Y	10	3	4	7	8	9	9	9	10	10	
9 M	10	1	2	2	2	7	9	10	10	10	
18 Y	10	0	0	0	0	0	0	0	5	10	
18 M	10	0	0	0	0	0	1	3	8	9	

¹ Y — young leaves only. Leaves approaching “full size” were removed from these plants periodically.

M — growing points removed from the above-ground parts of the plants starting January 9. 6–8 mature leaves allowed to remain on each plant.

² Short photoperiods (9 hr.) were applied on January 9 and the plants were returned to long days on February 15. Number of days for tuberization are counted from January 9.

were grown under several daylength and pruning regimes described in Table 4. All plants were subjected to long days prior to the start of the experiment. Tubers formed earliest on those plants that received the 9-hr. photoperiod. Within the short photoperiod treatment, however, those plants from which all older leaves (9Y) had been removed tuberized first, in spite of a probable low carbohydrate condition. With the exception of two aberrant plants, those plants from which the growing points were removed, leaving only mature leaves (9M) tuberized much later.

Plants of treatment 18M developed numerous extremely long vegetative stolons. Many of these stolons were 1–2 m. long. Stolons from plants in treatments 18Y and 9M were shorter, usually 0.5–1 m. long. Growing points of these very long stolons frequently pushed out from under the tank cover

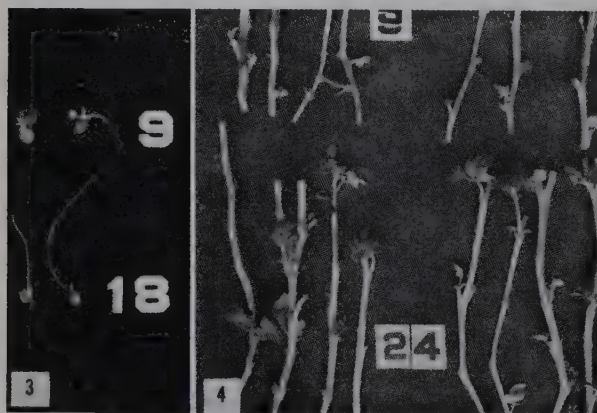


Figure 3. *Growth of nodes excised from short-day (9-hr.) and from long day (18-hr.) stems when placed on nutrient medium. Picture taken 11 days following transfer to culture flasks.*

Figure 4. *Tuberization of stem cuttings placed in a rooting bench. 9-stem cuttings from plants given a short daylength (9 hrs.) 24-stem cuttings from plants given a long day (24 hrs.).*

Table 5. *Tuberization of cuttings from plants exposed to 9- and 24-hour daylength.*

Treatment ¹	9-hr. daylength		24-hr. daylength	
	Number of cuttings	Number tuberized	Number of cuttings	Number tuberized
Basal - 1 node	6	6	6	0
2 nodes	7	7	7	0
3 nodes	6	5	7	0
Center- 1 node	10	9	6	0
2 nodes	10	8	7	0
3 nodes	7	7	7	0
Apical- 1 node	14	4	11	0
2 nodes	14	4	13	0
3 nodes	11	5	11	0

¹ Cuttings containing 1, 2 and 3 nodes were taken from basal, center, and apical regions of short and long day potato stems. Examined 11 days after planting in the rooting bench.

where they may have exerted an influence on tuberization. Tubers formed on stolons usually less than 6 cm. long in treatment 9Y.

Experiments with nodes grown in sterile culture. — Nodes from induced stems developed a tuber at the axillary bud following transfer to nutrient media while nodes from long day stems developed a vegetative shoot (Figure 3). The tubers were always 1 cm. or less in diameter and were frequently accompanied by a stolon-like development. Later many of these tubers grew out forming a vegetative shoot from the apical cluster of eyes indicating a loss of the tuber-inducing stimulus. This reversion to vegetative condition is frequently observed in commercial potato fields.

Tuberization of stem cuttings. — Within 10 days cuttings from induced stems formed tubers at the lower nodes when planted in moist sand. Stem cuttings from non-induced plants formed vegetative shoots from above-ground axillary buds, usually near the apex of the cutting (Figure 4). In another experiment cuttings with 1, 2, and 3 nodes from induced and non-induced plants were planted below the surface in a gravel-filled bench (Table 5). The cuttings from stems of induced plants again formed tubers while those from long day stems did not regardless of the number of nodes involved.

Discussion

The results of our experiments with grafted Triumph potato stems, tuberization of cuttings in sand or tissue culture, and short day cycle requirements for stimulus formation are essentially similar to those of Gregory (1951), who worked with Kennebec variety. In contrast however, Gregory states that

rhizome elongation as well as tuber formation is dependent upon inducing conditions, *i.e.*, short days and low night temperatures. In our work, length of the stolons is dependent upon vegetative vigor of the plants and the time at which tubers are initiated. Following induction of plants, tubers are differentiated at the growing points on the rhizomes or stolons. The stolons may vary from a few mm. to over 2 m. in length, thus resulting in a variation from only a few to well over 100 tubers being initiated on some plants.

A quantitative relationship seems to exist between the duration of induction and the amount of the tuber-inducing factor. A reversion to the vegetative condition apparently occurs if insufficient short-day cycle are applied even though some tubers may have started to form. This reversion may be manifest by renewal of stolon growth and "growing out" of tubers, and by renewed root growth and stem elongation. "Growing out" of tubers, observed many times in commercial fields, has been attributed to several factors including high soil temperatures, drought, or psyllid injury. Results from these experiments indicate the need for a continuous supply of the tuber-inducing stimulus following tuber initiation.

The grafting experiments clearly show the tuber-inducing stimulus is a transmissible factor. A union of tissues however, is essential. Movement of the stimulus is predominantly basipetal with little or no lateral movement, tuber formation always occurring first at the base of potato plants or of cuttings. The unilateral development of tubers and stolons on differentially treated stems of the same plant supports the conclusion that lateral movement occurs slowly. Serious damage to the vascular and cortical tissues of stems in the field cause tubers to form immediately above such damaged areas.

The tuber-inducing stimulus seems to be elaborated largely at active above-ground growing points. Placing only the growing points on an inducing day-length caused tubers to form. Furthermore, we have observed that a tuber will form in the terminal growing point of shoots held in sterile culture at 5°C. for several months, suggesting that the stimulus may be produced in this tissue but, under very low temperature conditions, is not translocated basipetally. In addition, Werner (1954) reported tuber formation in potato flower clusters which are born terminally on the plant. The failure of short-day stem cuttings from apical portions of plants to tuberize as readily as those from center or basal portions (Table 5), can be explained by the fact that axillary buds of such cuttings are frequently poorly differentiated and the stems are less fibrous and smaller in diameter, thus they grow less readily than fully differentiated buds and are more subject to breakdown by microorganisms.

The experiments with cuttings and nodes grown in sterile culture support

the conclusion that the tuber-inducing stimulus is not confined to the lower portions of the stem and to the stolon, but is present throughout the plant. Every leaf axil is capable of differentiating a tuber when properly cultured. This conclusion is further supported by the work of Werner (1954) who has demonstrated the formation of tubers at nearly any massed meristematic region except root tips within the plants of several varieties and seedling lines of potatoes. Tubers formed at the base of these plants were continually removed to force flower development. Several commonly observed growth responses indicate that a tuber-inducing stimulus may be present in the seed tubers under certain, as yet poorly defined, conditions. Seed pieces that are slowly destroyed by rot-producing organisms will occasionally form new tubers directly. Seed pieces of some varieties, *e.g.*, White Cloud, will sometimes form stolons and set tubers before sprouts emerge from the ground. Removal of several groups of sprouts from tubers, frequently results in new tubers being formed directly. In fact Weiss and Brierly (1926) have reported environmental conditions favoring tuber development directly from mother tubers.

While certain of the results can readily be explained on the basis of carbohydrate-nitrogen relationships, the proposed tuber-inducing stimulus or growth-substance theory more nearly fits the reported facts. All plants were grown in a common nutrient solution which eliminated the possibility of differences in mineral supply. The experiments were conducted during the fall and winter months when natural daylight of high intensity occurs for 9 hr. or less each day in the greenhouses. Thus both long- and short-day plants received nearly equal quantities of high intensity light, which is most effective for high rates of photosynthesis. Supplemental light to extend the daylength did not exceed 500 f.c. intensity and usually was less than 100 f.c. at the tops of the plants, being still less on the lower leaves. The limited quantity of photosynthate developed at these low light intensities (Chapman, 1953) would have little effect on carbohydrate-nitrogen ratios, especially since only 14 short-day cycles were sufficient to bring about tuber formation. The rapid transmission of the stimulus through grafts and the limited enlargement of tubers from nodes on culture media also shed doubt on the validity of the carbohydrate-nitrogen concept as a primary cause of tuber initiation.

Little is known about the chemical or physical properties of the tuber-inducing stimulus, in fact, several distinct substances may be involved. Probably it is formed when the plant is subjected to one of the so-called inducing conditions such as short daylength, but conceivably the factor is sometimes present in seed tubers and is inactivated or diluted by heavy vegetative development when plants are subjected to a long day. Formation of sprout tubers directly from seed pieces with no vegetative development favors such an

hypothesis. Thus far, however, we have failed to stimulate tuber formation at non-induced nodes by incorporating tuber extracts into White's medium.

Summary

A tuber-forming stimulus appears to be formed by active growing points or entire Triumph potato plants subjected to short-day conditions. Approximately 14 short-day cycles (9-hr. photoperiod) are required for the plant to form tubers. The stimulus readily passes a graft union causing non-induced stocks to form tubers. The stimulus is present throughout the plant, but moves principally in a basipetal direction as indicated by basal tuber formation on plants and cuttings. Differential daylength treatment of plants with two stems resulted in unilateral tuber formation on the short-day half of the stolon system.

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Effects of Photoperiod and Hormone Treatment on Isolated Rooted Leaves of *Kalanchoe Blossfeldiana*

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Introduction

Nearly all investigations into the effects of photoperiod on plants have been carried out with entire plants, and only on a few occasions have attempts been made to subject separated parts of plants to photoperiodic treatments (Lona, 1949, Carr, 1953, Zeevart, 1957). But even in these experiments it was necessary to use whole plants as test objects in order to detect any changes occasioned by inductive daylength treatment. Usually, this was done by grafting the isolated organs back onto a non-induced control plant.

These experiments are of considerable importance in view of the hypothesis put forward by Gregory (1948) that in the course of the production of the final flowering stimulus in short day plants, a photolabile leaf factor must first be translocated to the apical growing point of the plant where further reactions take place stabilizing this precursor. Hence the presence of an apical growing point is essential to this scheme. Those investigations in which detached organs were given inductive daylength treatments and subsequently grafted back, were designed to supply evidence regarding the locus of the stabilizing reactions of the flowering stimulus. A positive result would indicate that such stabilization can occur in the absence of translocation to an apex.

It seemed of interest in this connection to carry out some experiments with detached organs which would themselves (by morphological changes) indi-

cate whether photoperiodic treatments had caused changes without the necessity of test-grafting. For this purpose the leaves of *Kalanchoe Blossfeldiana* appeared very suitable material. As is well known from the researches of Harder and co-workers (Harder, 1948) the degree of succulence of *Kalanchoe* leaves is determined to a considerable extent by the daylength conditions under which they are grown, short-day conditions causing a high degree of foliar hydration accompanied by increase in thickness. Moreover, this effect is not confined to such leaves as are initiated and developed in short day, but older leaves at, or near, maturity also respond in the same manner.

It seemed of interest therefore to discover whether this morphological response to daylength in the leaves themselves could be provoked in the absence of any stem tissue and apical growing point.

Chibnall (1953) has shown that the act of detaching a leaf from the parent plant causes a considerable degree of protein hydrolysis in the leaf, but this can largely be re-synthesised when such a leaf is allowed to root. It was deemed likely therefore that the response of detached *Kalanchoe* leaves — if any — was likely to be better if they were allowed to root before treatment.

Methods and Results

1. Daylength Effects on detached, rooted leaves

In a preliminary experiment mature leaves taken from long-day grown plants were rooted in long-day. The petioles were cut well above the axillary buds and were then inserted into moist sand in a nearly saturated atmosphere, the leaf blades being upright. No hormone treatments were given. This method of rooting was used in all subsequent experiments. One group of these leaves was transferred to an eight-hour short-day some time subsequent to rooting; others received a sixteen-hour long-day as controls. Several months later their thickness was determined from sections cut across the leaf about half-way along its length. The mean thickness of the blade in short-day treated plants was $3.32 \text{ mm.} \pm 0.11$, while the long-day controls measured only $2.18 \text{ mm.} \pm 0.096$. Hence, the well-known effects of length of day on leaf thickness could also be reproduced in leaves detached before short day treatment.

In order to characterize these effects more closely, a similar experiment was set up in which more detailed measurements were taken. While the controls were maintained throughout in long-day, another group of rooted leaves was transferred to an eight hour short-day. At the beginning of this experiment a preliminary sample of the rooted leaves was taken, before differential treatment started. Fourteen weeks later all the leaves were harvested and the following data were recorded: lengths, widths, areas, thickness, fresh weight, dry weight, and water content as % dry weight. (Another group of leaves was also exposed to short-day conditions but with a 6-minute light break of low intensity (about 10 ft.c.) in the middle of the dark period. Unfortunately the amount of light given was too low (Harder and Bode, 1943) to modify the short day response significantly.)

It was noted also, that at the end of the experiment the leaves were no longer flat

Table 1. *Daylength effects on detached rooted leaves.*

Treatment	Length (cm)	Width (cm)	Area (sq. cm)	Thickness (mm)	Fresh weight (g.)	Dry weight (g.)	Water content (% dry wt.)
Preliminary sample	6.12	3.75	15.7	1.48	1.61	0.125	1185
Short Day	8.82	5.22	32.6	4.38	10.44	0.639	1534
Long Day.....	8.48	5.46	32.1	1.73	6.92	0.698	905
Sig. diffe.	N. S.	N. S.	N. S.	0.55	2.41	N. S.	160
Percentage increase after preliminary sample.							
Short day.....	44.1	39.2	107.5	195.9	548.4	411.2	29.5
Long day.....	38.6	45.6	104.3	16.2	329.8	458.4	-23.6

as they had been initially but showed considerable curvatures in both longitudinal and transverse directions. The degree of this curling was determined in a second experiment, *cf.* below.

The data have been collected in Table 1, in the second half of which the final values of the characters measured have also been expressed as the percentage of the initial values at the time of rooting the leaves.

The results indicate that in both long-day and short-day the rooted leaves approximately doubled their area during the experiment. This growth in area was due to approximately similar increases in length and breadth of blade (about 40 per cent in each direction). Treating the leaf as an ellipse the percentage area increases can be predicted from the percentage increases in length and breadth; and the predicted values are close to the actual figures indicating that no change in shape has taken place (*e.g.* for long-day leaves: predicted 101.8 per cent, actual 104.3 per cent). Thus there is little difference between the effects of long-day and short-day as regards cell expansion (no cell division occurs, *cf.* below) in the plane of the leaf. But the position is quite different in the direction at right angles to this plane. Here the increase in long-day was barely 16 per cent while the short-day leaves nearly trebled their thickness. The final dry weights were similar in both daylengths of approximately five times their initial weights. The percentage water content rose by approximately a quarter in short-day and fell by the same amount in long-day. Moreover the leaves were no longer flat but had curled considerably. This must have been due to somewhat unequal growth in area on the two sides of the leaves.

2. Growth Hormone Treatments

The next experiment was designed to discover the effects of indole-acetic acid treatment on this daylength response of detached leaves. The hormone

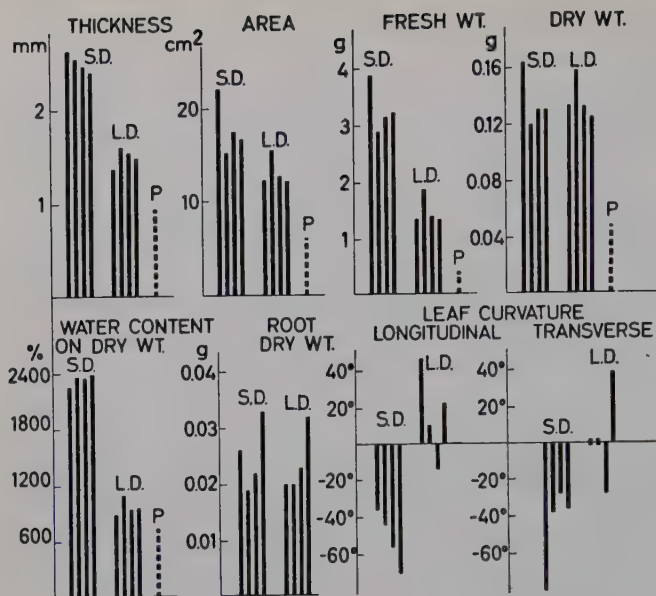


Figure 1. Effects of indole-acetic acid sprays on detached, rooted leaves grown in long- and short-day; left to right 0, 5, 50, 500 p.p.m. of IAA. Positive angular values indicate a convex curvature negative values concavity of the upper side. (SD = short days LD = long days P = initial value.)

was applied by spraying the leaves on both surfaces with an aqueous solution containing: 0.5, 50, and 500 p.p.m. indole-acetic acid. Six replicate leaves were used for each treatment. The leaves had been taken from L.D. plants and rooted in L.D. and then exposed to short- and long-day conditions for 20 days. At this time when the daylength effect were just becoming noticeable, the spraying treatment was begun, thirteen spray treatments being given in 21 days. After this time the leaves were harvested and a number of characteristics determined, these included: Thickness, area, fresh and dry weights, root dry weights and water contents. The angles of curvature of the leaves were also measured along the length and breadth, by determining the mid-points of the edges of the leaves and the midrib which yields an estimate of the angle subtended by the leaf at the centre of the circle of which it forms an arc (Figure 2A).

These data are presented in Figure 1, from which it is clear that the effects of auxin-spray treatment were negligible in comparison with the daylength effects, which were not modified significantly by the hormone applications. The daylength effects themselves are again large, short day treatment causing cell enlargement at right-angles to the plane of the leaf, as well as increases in overall area and per cent water content. Dry weights of the leaves and the dry weights of roots are identical and not affected by photoperiod.

The type of curvature differs with treatment, however. Short-day treated

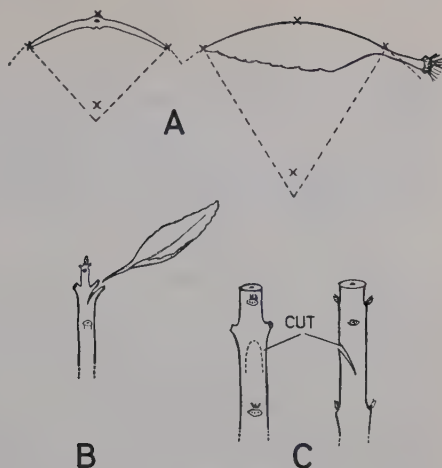


Figure 2. (A) Method of determining leaf curvature (angle \times). (B) and (C) Methods used in grafting leaves.

leaves have a concave upper surface forming an arc of 52° along their length and 46° along their breadth on the average of all short-day leaves, while long-day treated leaves are almost flat or slightly convex, exceeding 180° by an arc of 16° in a longitudinal and 2° in a transverse direction. Fig. 1 also records the average dimensions of leaves at the beginning of treatment (P). At the end of the experiment transverse sections were cut through all the leaves and counts made of the numbers of cell layers a short distance from the midrib. No differences were found between any of the treatments, regardless of length of day or hormone concentration, the distinction from leaves initiated in S.D. remaining undiminished. The numbers of layers in this region were (with variations of about ± 1 in the palisade and mesophyll):

	Upper epidermis	Upper sub-epidermis	Palisade	Mesophyll	Lower sub-epidermis	Lower epidermis
Rooted leaves in short day, or long day, or attached leaves from long day plants	1	1	4	8	1	1
Attached leaves from short day	1	1	4	2-3	1	1

Since only small differences in size were induced by the daylength treatments in both epidermal and subepidermal cells, all the variation in leaf thickness is accounted for by the expansion of palisade and mesophyll cells in a direction at right angles to the leaf surface. These findings agree closely with those reported for attached leaves by Gümmer (1949).

A further experiment was set up to discover whether application of hormones in a lanolin paste might be more effective. In this experiment somewhat younger leaves, which had not yet quite attained the final size of attached leaves, were used. They

were again rooted in long-day and about one month after being cut were transferred to their final daylengths and the hormone treatments applied at once. These included indole-acetic acid and the so-called anti-auxin, triiodobenzoic acid. A high concentration of these substances was given so as to maintain a "flow" of hormone for some time. Six weeks later, the leaves were again treated with paste. In one group the paste was applied over the whole upper (adaxial) and in another over the lower (abaxial) surface. Lanolin paste and "no paste" treatments served as controls.

Within the first few days after application of the paste the indole-acetic acid treated leaves showed some degree of epinasty where the paste was on the upper surface. No other symptoms became obvious during the further course of the experiment. At its conclusion the area and thickness of each leaf were determined, and some cell counts made.

It is clear from the results presented in Table 2A that the application of paste had a detrimental effect on further leaf growth, increases in area and thickness being curtailed compared with the no-paste controls. This effect on area is also just significantly greater where the paste was applied to the lower leaf surface. Although the daylength effects are large and obvious, none of the hormone treatments differs significantly from the lanolin controls.

A similar small experiment was carried out using young leaves which had been initiated and grown in short day until they were detached and then rooted in long day. The object of the experiment was to see whether leaves which had been formed under short-day conditions could still respond to daylength after being rooted and whether this response could be altered by indole-acetic acid application. After being rooted in long-day, half of the leaves were transferred back to short-day, the others remaining in long-day; the upper surfaces of one half of each were then treated with auxin paste. From the results presented in Table 2B, it is clear that the final thickness attained by leaves initiated in short day is also capable of being affected by length of day at a relatively late stage, further short-day treatment resulting in thicker leaves. In this instance it appears that area increases are greater if long-day treatment is given. Auxin paste again failed to alter the daylength responses, though in this instance it significantly enhanced the normal short-day increase in thickness. There was no effect in long day, however.

In both these experiments some leaves were sectioned to discover whether anatomical changes had been caused by the hormone treatment. In those leaves derived from long-day, the number of cell layers approximately midway between the midrib and the edge was relatively constant with the usual epidermal and sub-epidermal layers of small cells, 4 layers of palisade and 6 to 7 of mesophyll cells. (The number of mesophyll cells increases slightly in the region of the midrib and decreases near the leaf margin). However, in all treatments where pastes were applied to leaves, areas of dividing cells were

Table 2. (A) *Effects of indole-acetic acid and triiodobenzoic acid in lanolin paste on rooted leaves in long- and short-day applied on upper or lower surfaces.*
 (B) *Effects of indole-acetic acid paste on leaves initiated in short-day, rooted in long-day and then maintained in either long- or short-day.*

(A)	Mean area in cm ² (all treatments)	sign. diffce.	Thickness of blade in mm. (paste treatments)
IAA paste	18.8		1.11
TIBA paste	18.5		1.30
Lanolin paste	18.4		1.32
No paste control	25.1	2.78	(1.73 S. D. control)
Short day	23.3		1.33
Long day	17.1	1.96	1.17
Upper surface	20.9		1.23
Lower surface	19.1	1.96	1.29

(B)	(all treatments)	sign. diffce.	(all treatments) sign. diffce.		
Short day	4.2		2.79		
Long day	8.1	2.55	1.34		0.51
			<div style="display: flex; justify-content: space-around; width: 100%;"> short day long day </div>		
No paste control	6.5		2.12	1.35	
IAA paste	5.7	N. S.	3.45	1.32	0.87

found. These originated in the subepidermal layer either on the upper or lower surfaces and appeared to function as a cork cambium, up to 8 or 9 cells having been cut off. In most instances these patches could be correlated with epidermal injury caused during the application of the paste, though in some cases the epidermis appeared to be intact in the fixed material, probably indicating a lateral spread from some minor injury not seen in the section.

In the leaves originated in short day (Table 2B) the division into palisade and mesophyll cells was much more arbitrary, since both layers were much elongated in a direction at right angles to the leaf surface, in all 8 to 10 layers of cells represented these tissues. Again a few small patches of cork cambium were noted on the paste treated surfaces. The hormone and day-length treatments had not altered the number of cell layers present.

3. Grafting experiments

In another group of experiments attempts were made to establish whether these daylength effects on detached leaves included the production of the complete flowering stimulus, capable of being translocated by grafting to plants kept throughout in the long-day conditions unfavourable to flowering.

In the first instance it was tried to graft such rooted leaves onto the petioles of the test plants. The results were however quite negative since no successful grafts were obtained by this technique.

In a second grafting experiment the leaves to be grafted were inserted into a cut made directly into the stem of the stock plant through the base of one of the youngest expanded leaves, usually about 2 cm. or less below the terminal growing point. All the younger leaves above the graft union were removed so as to exclude any inhibitory effects due to these and to allow the normal upward translocation of the flowering stimulus to the terminal growing point to take place (Figure 2 B). Rooted leaves which had been maintained in short-day for several weeks were used together with three types of control: Rooted leaves maintained in long-day, leaves given short-day while attached to an entire plant, and leaves attached to a long-day treated plant. The graft combination was kept in 16 hour long-day throughout. Out of ten replicates per treatment the numbers of successful grafts were:

Rooted in short day	8	Rooted in long day	7
Attached in short day	9	Attached in long day	9

When the experiment was terminated ten weeks after making the grafts, not a single one of the receptor plants was flowering either at the terminal growing point or in a lateral axil, whether the leaf used as scion had been short-day induced while rooted or while attached to a plant. The failure to obtain transmission of the stimulus from the latter type of leaf (which is known to form the flowering stimulus) therefore ruled out any discrimination between the capacities of forming the flowering stimulus between attached and non-attached leaves.

In view of the many successful graft transmission experiments reported in the literature with other species and even with *Kalanchoe Blossfeldiana* (Carr and Melchers, 1954), a further attempt was made to obtain such transmission using a modified technique. Also, a further control treatment was added, namely the grafting of whole inflorescences derived from plants flowering after short-day induction, and having a number of succulent leaves.

The other four treatments consisted as before of rooted and attached leaves from long-day or short-day. The grafts were made by inserting the leaves or shoots into slanting cuts made into the side of the stem between two lateral buds in the same orthostichy (Figure 2 C). The main shoot was then cut off above the upper of these nodes. Most of the nodes both above and below the scions, carried only small axillary buds at the time of grafting. In one or two instances where somewhat larger leaves were present on them, these were removed so as to ensure the complete dependence of these buds for their carbohydrate supply on the rest of the plant. This was done because the suggestion has been made that the flowering stimulus is carried with the stream of assimilates.

Two and a half months after grafting, the condition of many of these 50 grafts was still very good (Table 3). None the less and in spite of all the precautions taken, there was once again an almost complete failure of trans-

Table 3. Condition of grafted leaves and shoots seven weeks after the grafting operation.
Ten replicates.

Type of graft	Completely healthy	Tip died back	Dead
Short day — rooted leaf.....	2	5	3
— attached leaf.....	5	1	4
Long day — rooted leaf.....	3	0	7
— attached leaf	6	2	2
Entire inflorescence with leaves	10	0	0

mission of the flowering stimulus. Even where whole flowering shoots were used as scions (with 100 per cent "take" of the grafts) only one of the receptor plants produced a single flower on the axillary branch above the grafted shoot. It would seem likely therefore that *Kalanchoe Blossfeldiana* is unfortunately not very suitable as a test plant for the transmissibility of the flowering stimulus by grafting, at least by the techniques used in the present experiments.

An attempt was also made to discover whether there was any transmission of the ability to fix carbon dioxide in the dark (Gregory *et al.*, 1954) Priestley (unpublished) established that a mechanism for the dark fixation of carbon dioxide was developed in such rooted short-day leaves in the same way as in whole plants exposed to short-day. However, he was unable to establish any transmission of this mechanism across the graft union in plants from the present experiment, either from grafted inflorescences, or from rooted leaves, although he found such transmission within a short-day induced plant from the older to the younger leaves.

4. Organic acid content

In a first attempt to correlate the morphological changes caused in such rooted leaves by differential daylength treatment with changes in their composition, analyses of their organic acid contents were made. Using the method of Pucher *et al.* (1941), the total acidity as well as the amounts of malic and citric acids present were estimated in rooted leaves exposed to short- and long-day conditions. Young and mature leaves attached to plants in both daylengths were also analysed at the same time. The results are presented in Table 4.

The total acidity has been expressed per unit fresh weight as well as per unit dry weight, the contents of malic, citric and other acids are given in terms of dry weight only. With some fairly large variation between treat-

Table 4. *Organic Acid content as c.c. N/100 acid of rooted and attached leaves.*

<i>Short day</i>	Total Acidity		Malic Acid per cm dry wt.	Citric Acid per gm. dry wt.	Others per gm. dry wt.
	per gm. fresh weight	per gm. dry weight			
Young attached leaves	18.9	467	191	8.5	267
Mature " "	14.0	463	240	9.3	214
Rooted leaves	24.1	442	75	3.8	363
<i>Long day</i>					
Young attached leaves	26.4	197	136	5.5	55
Mature " "	22.0	202	76	3.2	123
Rooted leaves	15.0	187	72	8.2	107

ments, the acidity expressed on a fresh weight basis are generally similar in short- and long-day, indicating similar acid concentrations in the cell.

On a dry weight basis, however, more than twice as much acid is present in short-day treated leaves. Of this rather less than half can be accounted for as malic acid and only a small amount as citric. Though in general the amounts may be said to be roughly proportional to total acidity, the differences between treatments in their contents of malic and citric acid are quite large. It would be of little use, however, at the present stage and without further experimentation to try to explain any of these differences.

On one occasion also pH determinations were made on the expressed sap of such rooted leaves, and these confirmed the generally higher acidity of the short-day leaves, the values being:

short day	4.35
long day	5.15

Discussion

Since Gregory's original suggestion (1948) that the complete induction reaction in short-day plants does not take place in the leaf and that it is not the final flowering stimulus which is translocated (as in grafts between induced and non-induced plants) but of a photolabile precursor, several attempts have been made to elucidate this question. The evidence for and against a photolabile precursor being stabilized only after transport to the growing point is rather conflicting. Carr (1953) has claimed that his failure to obtain induction by means of leaves induced in the detached condition could be interpreted as confirmation of Gregory's hypothesis. Unfortunately, he had no controls in which leaves from entire induced plants were transferred by grafting — a treatment which should have given positive results if any effects due to grafting were to be excluded. The failure to induce flowering in the receptor

might therefore still be interpreted as due to some specific grafting effect as such. Moreover, Chibnall's analytical data (1953) on protein hydrolysis of non-rooted detached leaves would also cast some doubts on the question whether such leaves could be regarded as functionally comparable with leaves attached to the plant.

Another method of attacking this problem was used by Lockhart and Hamner (1954) and also by Skok and Scully (1954) who determined in *Xanthium pennsylvanicum*, a plant which will flower after only a single long dark period, how soon after the end of dark treatment the induced leaf could be removed without interfering with the flowering of the plant. This method yielded some data on the rates of translocation which are much slower than would be required for the photolabile precursor to reach the growing point before the end of such a long dark period. Lockhart and Hamner estimated that a further 10 hours must elapse before such translocation had occurred, Skok and Scully's estimate also came to 10 hours.

The grafting experiments described briefly above have unfortunately failed to contribute to a solution of the problem whether the flowering stimulus is elaborated finally in the leaves of *Kalanchoe* before translocation to the growing point or not. Failure to obtain graft-transmission from rooted leaves induced by short-day can be interpreted neither as favouring such a hypothesis nor as opposing it, in the absence of any appreciable graft transmission from "plant-induced" leaves or whole inflorescences used as donor controls. Although it is known that graft transmission can be brought about in *Kalanchoe* also (Carr and Melchers, 1954) it clearly does not occur as readily as in some other plants (*e.g.* *Perilla*). The different methods of grafting, position of grafted shoots and leaves, as well as the usual defoliation techniques employed, all failed to ensure the flowering of the receptor plant to any significant extent, in spite of the fact that a high percentage of the grafted leaves had taken well. But in view of the accumulated data recording such transmission of the flowering stimulus it still seems likely that further changes of technique could overcome this difficulty.

Positive results such as Zeevart's (1957) with *Perilla* must therefore carry the greater weight, and strongly suggest that in *Perilla* at least the short-day reaction can become stabilized in the leaf.

The results with detached *Kalanchoe* leaves presented above are proof, however, that the reactions causing the morphological changes associated with short-day treatment in this species can be completed without the intervention of apical or stem tissue, unless one were to regard the root apices as functionally equivalent with stem apices. It is not clear, however, whether these reactions are identical with those leading to flowering. Harder and Gall (1945) believe that such morphological effects and flowering responses can

be separated. Using an anaesthesia technique during short-day treatment, they obtained increased leaf succulence without simultaneous flowering. On the basis of this result they actually postulated a separate hormone causing morphological changes, *i.e.* metaplasin, although in general the conditions for its production appear to be identical with those for the short-day flowering stimulus. Indeed the differences found by them might perhaps be explained on the basis of effects on translocation. Effects of anaesthetics on translocation were described recently by (Bukovac *et al.*, 1956).

The actual changes found here in rooted leaves are of interest in themselves. Counts of the numbers of cell layers in transverse sections through the leaves have indicated that no cell division is involved in the expansion growth of such leaves, all increase in size being due to enlargement of existing cells. The direction of such enlargement appears to be largely daylength controlled. As was seen in all the results given, there is a considerable increase in leaf area after rooting regardless of daylength treatment. The final areas reached under these conditions much exceed those that would have been attained had the leaves been left on the plant — in fact in most of the experiments they were excised at a time when they had practically reached their normal full size. Similar examples referred to by Goebel (1908) include observations made already by Knight (1841). This aspect is of considerable interest since it points to some definite control of the rest of the plant (stem, growing point or young leaves) over the duration and extent of cell expansion in the maturing leaves (*cf.* Goodwin 1937). Although the effect is the opposite, the failure of young leaves of *Hedera Helix* to expand after being detached and rooted (Gregory and Samantarai, 1950) may also be interpreted as due to some control by the rest of the plant.

From the data in Table 1, it was seen that the percentage increases in cell size in all directions in the plane of the leaf are approximately equal, though this enlargement cannot be of equal magnitude in all the layers, as is shown by the curvature which develops in the rooted leaves. These differences are themselves affected by length of day treatment, leaves enlarging in short day have a relatively lower degree of cell expansion on the adaxial (upper) side, leading to concavity of that surface. Presumably it is the palisade tissue which undergoes the least enlargement in this plane. Some slight restraint appears also to be due to a more limited expansion of the marginal cells, which causes occasional cracks to develop in the leaf edges. Such leaves curl in an arc of about 50° from the flat position in both primary directions. Long-day treated leaves are much flatter or even convex.

The major effect of length of day on cell expansion is, of course, on the extension found in the direction at right angles to the plane of the surface, leading to changes in leaf thickness; in rooted leaves kept in short-days the

overall length of palisade and mesophyll cells increases by a factor of about 3. Similar anatomical effects in intact plants have been subjected to a close study by Harder and his co-workers (Gümmer 1949) and the present results with rooted leaves are in agreement with their findings.

These daylength effects were not modified by the application of growth hormone treatments. Indole-acetic acid applied as sprays in concentrations from 5 to 500 p.p.m. had no significant effects, and when applied in lanolin paste its effects did not differ from those due to lanolin alone. Similarly the so-called anti-auxin triiodobenzoic acid applied in lanolin paste did not alter the daylength responses. The fact that all paste treatments caused reductions in expansion of area and thickness can probably be explained by stomatal blockage on the treated surface and reduced assimilation etc. Such treatment also caused some epidermal injury as is evidence by the production of a cork cambium from the sub-epidermal layer of cells. It would seem unlikely therefore that indole-acetic acid is directly concerned in this daylength effect.

The preliminary analyses for organic acids in the leaves reveal much greater contents per unit dry matter and also amounts per leaf, in short day conditions. Their concentration is much less affected by daylength, however, owing to the very high water contents of short-day treated leaves. It would be of very considerable interest in this connection to discover whether there is any causal relation between high acidity and succulence or whether these are parallel effects caused by other changes. The pH of the cell sap of the short-day leaves was also lower by as much as 0.8 of a unit than that found in rooted long-day leaves. The differences between the relative amounts of malic and citric acids do not reveal any clear pattern and in any case these two acids represent only about half the total acidity found.

Priestley's finding (unpublished) that another metabolic effect associated with flowering, *i.e.* the mechanism for enhanced dark fixation of CO_2 , is also developed in such rooted leaves is of interest in relation to these results. Norris and Calvin's (1955) analysis of the radioactive carbon distribution after 5-minute photosynthesis in long- and short-day *Kalanchoe* plants also revealed some differences in the activities recorded in the acid regions of their chromatograms. The long-day set gave higher readings (as in nearly all other fractions) when these values were expressed on a fresh weight basis, but on a dry weight basis the short-day set again showed the higher activity. The diurnal cycle of acidity changes in Crassulacean metabolism and its light dependence have been investigated by Bennet-Clark and his school (Bennet-Clark 1949) as well as by Pucher *et al.* (*e.g.* 1949), while Thomas (1949) and Thomas and Beevers (1949) have closely studied the dark fixation mechanism. The field has been reviewed recently by Burris (1953). It may only be added here that the daylength regime to which the plant material has been sub-

mitted, also modifies this aspect of metabolism. Though nothing useful can be said at this stage concerning the reasons for the phenomena, nor even their precise nature defined, this aspect of photoperiodism deserves further study.

Summary

Detached leaves of *Kalanchoe* have been rooted without hormone treatment. Such leaves continued to increase in area for long periods and their final sizes much exceeded that which they would have attained if left on the plant. When differential daylength treatment was given, detached and rooted leaves in short-day responded by large increases in thickness, fresh weight and water content compared with long-day controls, in the same manner as attached leaves in short-day. All these increases were entirely due to cell enlargement, the number of cell layers remaining unchanged. The short-day effect was found to be due solely to excessive enlargement of palisade and mesophyll cells in a direction at right angles to the plane of the leaf. Indole-acetic acid spraying in concentrations ranging from 5 to 500 p.p.m. had no significant effect on these responses. Indole-acetic and triiodobenzoic acids applied in lanolin paste were also without effect, but all paste treatment including pure lanolin was detrimental. Grafting experiments using such leaves and non-flowering receptor stocks gave inconclusive results owing to failure of control leaves and even flowering shoots used as donor-scions to induce the receptor to flower. Analysis of the organic acid contents of short- and long-day treated leaves indicated higher levels in short-day on a dry weight basis, malic and citric acids accounting for less than half the total acidity. Owing to the higher water contents of short-day leaves the concentrations of acid in the cell sap did not differ significantly from the long-day controls.

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Investigations of the Cytoplasmic Particulates and Proteins of Potato Tubers VI.

Nitrogen Changes Associated with Emergence of Potato Tubers from the Rest Period

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It was earlier shown by direct extraction (Levitt 1954) that protein synthesis in the internal tissues of potato tubers heralds the end of the rest period. In the absence of direct determinations, it was necessary to assume that this synthesis was at the expense of stored amino acids. In order to test this conclusion and to investigate the phenomenon still further, the soluble and total nitrogen have now been determined. The difference between these two (the insoluble N) is taken to represent protein N. In this way, the results of two different methods of analysis can be compared.

Methods

a. Preparation of tubers

As in earlier experiments, a 100 lb. sack of potatoes (Russet variety) was purchased each fall in 1955 and 1956 and stored at 3°C for the whole year's experiments. Only those potatoes weighing 200—250 g were used.

Tubers were taken at monthly intervals and cut in half longitudinally. One half was retained at 3°C, the other half transferred to 26°C for ten days. The halves were then peeled, sliced, rinsed in distilled water and blotted with paper toweling. The peel included the whole xylem layer and is called the external tissues; the remaining part which was then sliced is called the internal tissues.

The slices and peel (when not discarded) were separately frozen in liquid air then wrapped in cheesecloth and freeze-dried in a vacuum desiccator between layers of

80 mesh alumina. The dried slices were ground in a Wiley mill to pass a 60 mesh screen and the powder stored at 3°C. All the analyses were made after the last samples were taken in February. The dates in the tables refer to the time when the tissues were freeze-dried.

All the tuber halves kept for 10 days at 26°C began showing signs of sprouting in January. Consequently, this is taken as the time when the rest period was broken. The rest period in these experiments therefore includes the September to December analyses. In one case, signs of sprouting were visible in December.

b. Analytical methods

The soluble N was extracted from the potato powder with 80 per cent ethanol (volume per cent) at 3°C. Three gram samples were extracted with 25 ml. ethanol, filtered, and rinsed with 8—11 aliquots of 20 ml. until 200 ml. of extract was obtained. The extracts were dried in vacuo over alumina at 3°C for 2—3 days. The residue was then taken up in 10 per cent isopropyl alcohol to make 50 ml. These solutions were then used for free amino acid and soluble N determinations. When the amino acids were not determined, the ethanol extract was used directly without drying.

The Nessler method was used for total N and for alcohol soluble N, according to Lindner (1944). A Klett-Summerson colorimeter was used the first year, a Bausch and Lomb Spectronic 20 the second year. For total N, 88—100 mg. samples of potato powder were digested.

For alcohol soluble N, 4 ml. aliquots of the isopropyl alcohol, or 25 ml. of the more dilute ethanol extracts were used. Free amino acids were determined by the two-dimensional paper chromatographic procedure of Thompson et al. (1951). The ninhydrin spots were extracted with 10 ml. 50 per cent ethanol and the concentration determined on a Carey spectrophotometer. The blue spot extracts were read at 570 mμ, the yellow at 330 mμ.

Results and Discussion

I. Results obtained with internal tissues of tubers stored continuously at 3°C (Table 1—3)

In both years the alcohol insoluble N reached a maximum in January. The corresponding alcohol soluble N did not reach the minimum value, though it was definitely a minimum between December and February in the second year. These results agree in general with the protein results (Levitt 1952, 1954); in four of the five years, the protein maximum was reached during December or January (Table 3).

II. Tubers stored at 3°C from September, then half a tuber transferred to 26°C for 10 days at monthly intervals (Tables 1 and 2). Internal tissues

1. In both years, alcohol soluble N increased at the expense of alcohol insoluble N during October.
2. In both years, the reverse happened during December.

Table 1. *N* fractions (as % dry matter) in potato tubers during (September—December) and following (January—February) the rest period. Internal tissues 1955—56.

Date	Total N	Soluble N	Insoluble N
A. Stored continuously at 3°C			
October 26	0.78	0.31	0.47
November 26	0.86	0.51	0.35
December 26	0.92	0.54	0.38
January 26	1.17	0.56	0.61
February 26	1.13	0.63	0.50
B. Stored at 3°C then transferred to 26°C for 10 days			
October 4	0.78	0.41	0.37
October 26	0.76	0.33	0.43
November 26	0.88	0.39	0.49
December 26	0.85	0.38	0.47
January 26	1.19	0.66	0.53
February 26	1.12	0.63	0.49

Table 2. *N* fractions (as % dry matter) in potato tubers during (September—December) and following (January—February) the rest period. Internal tissues, 1956—57.

Date	Total N	Soluble N	Insoluble N
A. Stored continuously at 3°C			
October 10	1.25	0.38	0.87
November 12	1.31	0.49	0.82
December 10	1.49	0.88	0.61
January 10	1.68	0.66	1.02
February 10	1.52	0.97	0.55
B. Stored at 3°C then transferred to 26°C for 10 days			
September	1.37	0.73	0.64
October 10	1.27	0.76	0.51
November 12	1.35	0.79	0.56
December 10	1.46	0.82	0.64
January 10	1.62	0.95	0.67
February 10	1.62	0.98	0.64

Table 3. Protein *N* (as % dry matter) in internal tissues of potato tubers during continuous storage at 3°C.

Month	Extracted proteins					Insoluble N	
	'49—50	'50—51	'51—52	'52—53	'53—54	'55—56	'56—57
October	0.21	0.32	0.42	0.43	0.36	0.47	0.87
November	0.18	0.26	0.41	0.41	0.38	0.35	0.82
December	—	0.51	0.39	0.46	0.41	0.38	0.61
January	0.32	0.32	0.36	0.41	0.38	0.61	1.02
February	0.30	0.30	—	—	—	0.50	0.55

Table 4. *Per cent change in proteins in internal tissues of potato tubers on transfer to 26°C for 10 days after continuous storage at 3°C.*

Month	Extracted proteins			Insoluble N	
	'51—52	'52—53	'53—54	'55—56	'56—57
October	— 1.0	+ 3	— 5	— 9	— 41
November	— 10.5	+ 3	+ 11	+ 40	— 39
December	+ 20.0	+ 10	+ 10	+ 24	+ 5
January	+ 1.5	+ 15	0	— 13	— 34
February	— 11	— 8	— 14	— 2	+ 16
March	— 10	— 6	—	—	—
April	— 5	—	—	—	—
May	— 7	—	—	—	—
June	—	—	—	—	—
July	— 8	—	—	—	—

3. Both of these results agree with three previous years' direct determinations of proteins (Table 4) which showed a maximum net protein synthesis in December, little or none in October.

Both sets of results corroborate the conclusions arrived at earlier (Levitt 1954). Not only do the two distinct methods of determining protein N (direct extraction of proteins and determination of alcohol insoluble N) yield the same results, but even the actual change in alcohol insoluble N on transfer from 3°C to 26°C is quantitatively accounted for by the reverse change in alcohol soluble N (Table 5). This gives direct proof of the assumption (Levitt 1954) that the proteins are synthesized or broken down at the expense of or with the production of amino acids or amides. Direct determinations showed that the major changes were in the amides, particularly asparagine (Cotrufo 1957). Whenever large changes in soluble N occurred, these were accounted for by the changes in amides (Table 6).

In contrast to the above balance between soluble and insoluble N, and the

Table 5. *Changes in soluble and insoluble N (% of dry matter) on transfer from 3°C to 26°C for 10 days.*

Month	1955—56		1956—57	
	Insoluble N	Soluble N	Insoluble N	Soluble N
October	— 0.04	+ 0.02	— 0.36	+ 0.38
November	+ 0.14	— 0.12	— 0.26	+ 0.30
December	+ 0.09	— 0.16	+ 0.03	— 0.06
January	— 0.08	+ 0.10	— 0.35	+ 0.29
February	— 0.01	— 0.00	+ 0.09	+ 0.01

Table 6. *Changes in soluble N and in amides (asparagine plus glutamine) during 10 days at 26°C.*

Date	Change in soluble N	Change in amides
October 26, 1955	+ 0.02	— 0.03
November 26	— 0.12	— 0.11
December 26	— 0.16	— 0.10
January 26, 1956	+ 0.10	+ 0.09
February 26	0.00	+ 0.03

lack of change in total N during 10 days at 26°C, the tubers kept continuously at 3°C showed a lack of agreement, due to an increase in total N of the internal tissues from October to December. That these increases are significant is indicated by the close agreement between the total N of corresponding halves of the same tuber kept at 3°C and 26°C for 10 days (Table 7). It is, of course, to be expected that differences between tubers would be greater than between corresponding tuber halves; but the similar steady rise during two successive years is very unlikely to be due to such chance variations. Further evidence that these differences are real, due to transfer of soluble N to or from the external tissues, is indicated by the fact that the results of the two years fall on the same curves (fig. 1).

These N changes both at 3°C and at 26°C can be explained by the following working hypothesis:

Sprouting depends on a synthesis of proteins in the buds. This in turn depends on a movement of amino acids to the buds from the internal tissues where they are stored. When the tuber is in the rest period, this movement is stopped (a) by protein synthesis in the internal tissues and (b) by an actual reversal of movement of amino acids from the external into the internal tissues. These two processes are partially independent. Only when both processes lead to a movement toward the external (bud containing) tissues is the tuber capable of rapid active growth.

Table 7. *Total N (% of dry matter) of potato tuber halves kept continuously at 3°C compared with corresponding halves transferred to 26°C for the final 10 days.*

Month	1955—56		1956—57	
	3°C	26°C	3°C	26°C
October	0.78	0.76	1.25	1.27
November	0.86	0.88	1.31	1.35
December	0.92	0.85	1.49	1.46
January	1.17	1.19	1.68	1.62
February	1.13	1.12	1.52	1.62

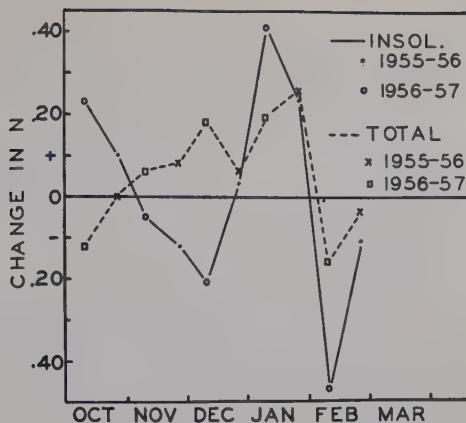


Figure 1. Changes in total and insoluble N of internal potato tuber tissues during storage at 3°C. Expressed as % of dry matter.

The results obtained above can be seen to agree with this hypothesis. Thus, when the tubers are transferred to 3°C shortly after harvest (late September or early October) they are in their rest period. Though the amino acids are moving outward (favoring growth), this is counteracted by a synthesis of proteins in the internal tissues. These two processes combine to reduce the amino acid content of the internal tissues to a minimum in October. The outward movement of amino acids soon comes to a stop and is then reversed (early November). From then until late January, an inward movement of amino acids continues at an almost steadily increasing rate. The initial protein synthesis continues, though at a decreasing rate, for about a month after transfer to 3°C. At about the same time as the reversal of amino acid translocation (Nov. 10), there is also a reversal from protein synthesis to hydrolysis. This hydrolysis continues for another month but cannot favor active growth because the amino acids are moving inward. By the end of December, the hydrolysis has again reversed to a protein synthesis which accelerates to a maximum around January 10. At this point there is a precipitous reversal to hydrolysis, and the amino acids pile up to a maximum. This is soon followed by an equally precipitous reversal of amino acid movement to an outward direction. The simultaneous occurrence of these two processes is accompanied by complete breaking of the rest period.

High amino acid content in the internal tissues is thus associated with deep rest, high protein with the end of the rest period. This may imply that the changes in the external tissues are similar to those in the internal tissues, and the buds cannot sprout until an active protein synthesis occurs. Thus, when protein synthesis is at a maximum in the internal tissues, the tubers are capable of showing just a trace of sprout formation if transferred to 26°C for 10 days (Levitt 1954), perhaps due to a starvation for amino acids which

Table 8. *N* fractions (as % dry matter) in potato tubers during (September—December) and following (January—February) the rest period. External tissues 1956—57.

Date	Total N	Soluble N	Insoluble N
A. Stored continuously at 3°C			
October 10	1.05	0.36	0.69
November 12	1.08	0.25	0.83
December 10	1.22	0.43	0.79
January 10	1.35	0.38	0.97
February 10	1.21	0.41	0.80
B. Stored continuously at 3°C then transferred to 26°C for 10 days			
September	1.24	0.22	1.02
October 10	0.97	0.24	0.73
November 12	1.08	0.22	0.86
December 10	1.21	0.39	0.82
January 10	1.35	0.45	0.90
February 10	1.11	0.44	0.67

are not moving out of the internal tissues. It is only in January and later that the sprouts become large during 10 days at 26°C (Levitt 1954). At this time, proteins are being hydrolyzed in the internal tissues and amino acids are moving out to the buds. Transfer to 26°C for 10 days tends to speed up the process. For this reason, maximum protein synthesis is usually attained in December at 26°C instead of January (in the case of tubers maintained continuously at 3°C).

It should be possible to test this theory by a simple analysis of the external tissues. The total N changes in these external tissues should be the mirror images of those in the internal tissues. One year's results fail to show any such relation (Table 6). But simple calculations show that a loss of N from the external to the internal tissues could be completely masked by a concomitant loss of carbohydrates. Thus if the external tissues comprise 25 per cent of the tuber and if 25 per cent of its total N and 50 per cent of its digestible carbohydrates were translocated to the internal tissues (assuming that 75 per cent of the total carbohydrate is digestible), the internal tissues would show a 3 per cent increase in soluble N and the external tissues instead of decreasing, would show an increase of 30 per cent in soluble N as a percent of dry matter. (The insoluble N and the soluble N are each assumed to be 0.50 per cent of dry matter at the start.) If instead of 50 per cent, 33 $\frac{1}{3}$ per cent of the carbohydrates were translocated from the external to the internal tissues, the soluble N of the internal tissues would increase by 13 per cent, that of the external tissues would show no change. It is thus impossible to check the hypothesis by a simple analysis of the N content of the external tissues.

On the other hand, certain differences between the internal and external

tissues do uphold the above hypothesis. At both temperatures, but especially at 26°C, the external tissues have half or less than half the soluble N content of the internal tissues, though the insoluble N is about the same in the two. This agrees with the concept of the internal tissues acting as a N reserve for the external tissues. In further agreement is the large conversion of insoluble to soluble N in the internal tissues on transfer to 26°C for 10 days (e.g. in October and November). The external tissues fail to show any conversion.

It does not necessarily follow that exactly the same cycle of changes must occur in all years, particularly since the tubers were simply purchased and nothing was known of the preceding treatments they had been subjected to. Emilsson (1948) has reported that many factors may significantly alter the rest period, *e.g.*, maturity at time of harvesting, temperature, etc. One exceptional year's results (1951—52) has already been recorded (Levitt 1952). This can perhaps be explained by the high N content. Thus in 1956—57, the high total N content during October was due to a higher protein content than 1955—56. The amino acid content was about the same in the two years. In view of the already high protein content, it is not surprising that the net protein synthesis during December was small (1956—57) or even undetectable (1951—52). When, on the other hand, the tuber entered its rest period with a low protein content (1953—54 and 1955—56), a net synthesis occurred in November as well as December and the net was large.

How do the results of other investigators fit in with this working hypothesis? Newton (1927) found a slight decrease in the amino N during the first four weeks of storage at 5°C, followed by an increase to about the initial value at the end of the rest period. This agrees with the results described above except that the increase went beyond the initial value. Emilsson (1949) analyzed tubers four times from October to March and found a slight increase in protein N during December and January and a corresponding decrease in soluble N. This again agrees with the above results. But neither of these investigators (nor others) separated the internal from the external tissues and therefore their results are not strictly comparable to those reported here.

Summary

1. Internal tissues of tubers stored at 3°C from September or October showed a maximum insoluble N content in January.
2. On transfer of these tubers to 26°C for 10 days, alcohol soluble N increased at the expense of alcohol insoluble N during October, while the reverse happened in December. These changes were accounted for by the changes in the amides asparagine and glutamine.

3. Both the above results agree with several years' results obtained by direct extraction and determination of proteins.
4. Total N of the internal tissues increased from October to December, due presumably to a translocation of N from the external tissues.
5. A working hypothesis is proposed to explain these results. N determinations were made on the external tissues, but these are incapable by themselves of yielding information needed to test this hypothesis, if carbohydrate translocation also occurs.

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Die Entwicklung von *Cannabis sativa* unter dem Einfluss verschiedener Tageslängen

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Entwicklungsphysiologisch ist der Hanf bisher vorwiegend unter dem Gesichtspunkt der Geschlechtsausprägung untersucht worden. Doch sind in den Veröffentlichungen meistens auch einige Beobachtungen über seine vegetative Entwicklung mitgeteilt. Ausführlichere Angaben machen drei Autoren: Petit (1952) untersuchte den Einfluß der Tageslänge auf die Ausbildung der Sproßachse und stellte fest, daß die Internodien im Kurztag eine geringere Länge erreichen als im Langtag. Nach einem Wechsel der Tageslänge erhalten die neugebildeten Internodien eine der herrschenden Tageslänge entsprechende Länge. Die verkleinernde Wirkung des Kurztags erstreckt sich auch auf Blattstiele und Blätter. Die allgemeine Formveränderung der Blätter während der Entwicklung, nämlich stärkere Teilung der Blätter und Vermehrung der Blattrandzähne mit zunehmender Insertionshöhe und Umkehr dieser Entwicklung nach Beginn der Blüte, wird durch Kurztag beschleunigt: Das erste 5-Blättchenblatt entsteht an niedrigeren Knoten als im Langtag. Andererseits wird im Langtag eine höhere Blättchenzahl erreicht, auch die Blattzahl vor der Blüte ist im Langtag um das Mehrfache erhöht. Heslop-Harrison (1956) beschreibt die gesetzmäßige Veränderung der Blattform unter dem Einfluß verschiedener Tageslängen mit Hilfe eines Blattformindex, der sich aus dem Produkt der Zahl der Blättchen eines Blattes und der Zahl der Zähne an einem Rand seines Mittelblättchens ergibt. Lange vor beiden Autoren berichtete Schaffner (1926/28) über die Wirkung einer Verlängerung des Tages nach Beginn der Blüte. Hanfpflanzen, die in der Blütenregion mehrfach geteilte und stark gezähnte Blätter besaßen, bildeten im Dauerlicht neue

Blätter aus, deren Form sich immer weiter von der der im Kurztag gewachsenen Blätter entfernte, sie waren immer weniger differenziert und schließlich ungeteilt und ganzrandig, kotyledonenähnlich. Während dieser „rejuvenation“ wurden auch keine Blüten mehr ausgebildet. Nach Abschluß der Verjüngung begann noch im Langtag ein neuer Differenzierungszyclus und nach neuerlicher Verkürzung der Tageslänge eine neue Blühperiode. Durch weitere Langtags- und Kurztagsgaben erlebten einige Pflanzen vier vollständige Entwicklungszyklen.

Alle diese Beobachtungen über die Veränderung der Blattform konnten wir bestätigen, doch ist mit der Blattform nur ein, wenn auch wesentlicher Teil der Entwicklung erfaßt. Zur vollen Charakterisierung muß auch die Blattgröße berücksichtigt werden. Sie ist im Gegensatz zur mehr endonomen Blattform viel stärker von Außenfaktoren abhängig. Erst beide Eigenschaften der Blätter ergeben ein vollständiges Bild, das Schlüsse auf die den Veränderungen zugrunde liegenden physiologischen Vorgänge zuläßt. In der im Folgenden beschriebenen Untersuchung wurden beide Faktoren berücksichtigt.

Versuchsanstellung

Am 14.2.1956 wurden Samen der Hanfsorte Pelozella in Petrischalen gequollen und die gekeimten vom 15. bis 20.2. in Töpfe von 6 cm Ø mit Komposterde gepflanzt. Die Aufzucht erfolgte bis zum 13.4. in einem behelfsmäßigen Klimaraum unter Leuchtstoffröhren (Philips TL 40) in einer von 16 auf 24 Stunden schrittweise verlängerten Tageslänge bei mindestens 15°C. Zu diesem Zeitpunkt war das dritte Blattpaar (Keimblätter nicht mitgezählt) voll ausgebildet. Die Pflanzen wurden in größere Töpfe (Ø 9 cm) umgesetzt und je 32 in 24, 16 und 10 $\frac{3}{4}$ Stunden-Tag (bezeichnet als 11) weiter herangezogen. (11 Std.-Tag durch Verdunklung und 24 Std.-Tag durch zusätzliches Licht mit Leuchtstoffröhren im Gewächshaus; 16 Std.-Tag in der erwähnten Klimakammer). Im 11- und 16 Std.-Tag kamen die Pflanzen zur Blüte, im Dauerlicht nicht. Der mittlere Blühtermin im 11 Std.-Tag war der 28.4. für ♂ oder 3.5. für ♀; im 16 Std.-Tag der 2.5. (♂) bzw. 12.5. Als Blühtermin einer Pflanze gilt der Tag, an dem entweder die erste Narbe oder die erste männl. Blüte mit Sicherheit erkennbar ist.

Am 11.5. wurde von den Pflanzen, die bisher Dauerlicht bekommen hatten, je $\frac{1}{3}$ im 24 Std.-Tag belassen oder in 11- und 16 Std.-Tag übertragen; am selben Tag wurden die Weibchen der 11 Std.-Gruppe auf die 3 Tageslängen verteilt. Die Weibchen der 16 Std.-Gruppe ebenso, doch wegen des späteren Blühtermins erst am 24.5. Insgesamt sind damit 9 Gruppen entstanden, die nach ihrer Behandlung bezeichnet werden, z.B. mit $\frac{24}{16}$ die Gruppe, die ab 13.4. Dauerlicht und ab 11.5. 16 Std.-Tag bekam.

Auswertung

Protokolliert wurde von sämtlichen noch vorhandenen Blättern jeder Pflanze in der natürlichen Reihenfolge ihrer Bildung die Anzahl der Blättchen, Länge und Breite

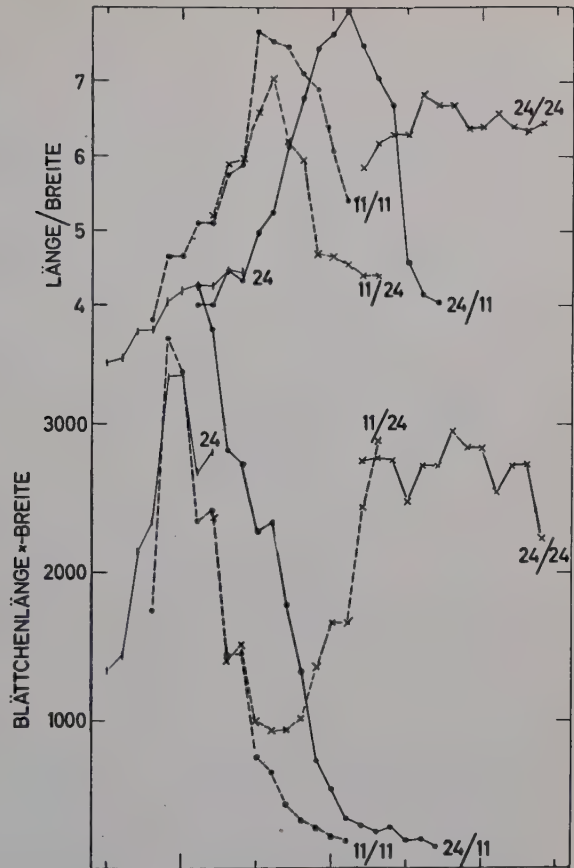


Abb. 1. Mittelwerte für Blattform (oben) und Blattgröße (unten) in verschiedenen Tageslängen.

des (größten) Mittelblättchens und die Zahl der Zähne. Im Folgenden werden nur die Maße der Mittelblättchen berücksichtigt. Das Produkt aus Länge und Breite ist ein Maß der Größe des Blattes, ihr Quotient ist ein Maß für seine Form. Die größeren Blättchen eines Blattes haben den gleichen Quotienten, aber natürlich verschiedene Größe. Jedes Blatt ist in Beziehung zu seiner Stellung an der Sproßachse gesetzt. Blatt 1 und 2 sind die ungeteilten Primärblätter, 3, 4 usw. die gefingerten Folgeblätter. Gegenständige Blätter bis zum 5. bis 9. Knoten haben nahezu gleiche Werte für Blattform und Größe. In Abb. 1 ist die Abszisse die natürliche Reihenfolge der Blätter, die Abstände zwischen den Ziffern sind gleichmäßig gewählt ohne Rücksicht auf die Internodienlänge.

Die folgenden Angaben beziehen sich stets nur auf weibliche oder vegetative Pflanzen. In Abb. 1 oben sind die Blattformwerte der Gruppen 11/11, 11/24, 24/11 und 24/24 eingetragen, zusätzlich eine Gruppe, die nur bis zum 9.5. Dauerlicht erhielt (24). Bei allen Gruppen steigen die Werte an; bei den Gruppen, die anfangs Dauerlicht erhielten, ist der Anstieg verzögert. Nach Errei-

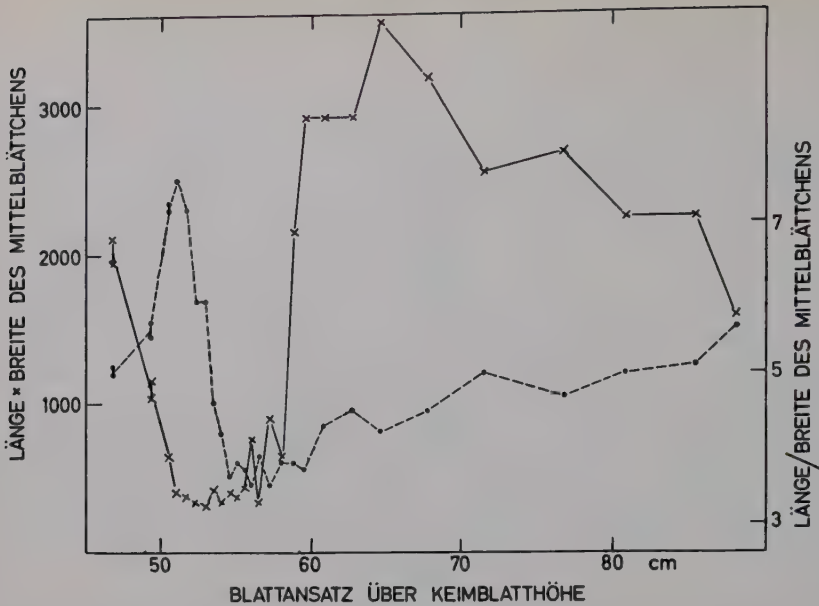


Abb. 2. Blattform- (·) und -größenwerte (×) vor und nach dem Ende des ersten Lebenscyclus bei einer Pflanze der Gruppe 11/16.

chen eines Maximus folgt ein Abfall der Werte bei den Gruppen, die zuerst oder später Kurztag erhielten, nur die Gruppe 24/24 zeigt einen solchen Abfall nicht; sie scheint einen Höchstwert erreicht zu haben und beizubehalten. Der Abfall der drei Kurven ist nicht sofort nach Kurztagsbeginn erfolgt, sondern mit einer gewissen Verzögerung; das ergibt sich aus dem Ort der Divergenz der Kurven.

Gleichzeitig mit der Verbreiterung der Blätter in oder nach Kurztag verläuft ein rein tageslängenabhängiger Vorgang. Dies zeigt der untere Teil der Abb. 1 mit den Werten für die Blattgröße derselben Blätter, deren Formwerte oben zu sehen sind. Die Kurven 11/11 und 24/11 zeigen einen kontinuierlichen Abfall. Nach Ausbildung der minimalen Blätter sterben die Pflanzen ab. Bei der Gruppe 11/24 schlägt der Abfall dagegen in einen Anstieg um. Nach anfänglicher Verkleinerung werden die Blätter fortlaufend größer. Schließlich werden Blätter ausgebildet, die sich von den Primärblättern eines Keimlings nur dadurch unterscheiden, daß sie größer sind. Sie sind relativ breit, ungeteilt und kaum gezähnt (s. Abb. 3).

Diese vergrößerten Altersblätter sind nun tatsächlich Primärblättern gleichwertig. Läßt man nämlich solche „verjüngten“ Pflanzen weiter in der Tageslänge, in der sie sich verjüngt hatten, so macht ihr Sproß einen zweiten Lebenscyclus über die Blüte zum Tode durch. Dies zeigt Abb. 2. Es handelt sich um

Tab. 1. *Extreme Blattformwerte verschiedener Herkünfte. (Verjüngte Pflanzen.)*
Mittelblättchen-Länge/-Breite.

Sorte	Blattpaar 7/8	Größter	Zweitgrößter	Kleinstes	Zweitkleinstes
F	3,45 (2)	5,14 (5)	4,92 (5)	2,53 (10)	2,95 (10)
F × P	—	6,08 (5)	5,35 (4)	2,41 (8)	2,68 (8)
C	—	—	—	2,25 (2)	2,50 (2)
C × P	—	—	—	2,33 (4)	2,58 (4)
P	—	—	—	2,69 (7)	2,99 (7)
P × B	—	—	—	2,52 (17)	3,01 (17)
J × B	4,82 (10)	7,51 (10)	7,14 (10)	4,24 (10)	4,71 (10)
	4,67 (10)	7,13 (10)	6,87 (10)	4,09 (10)	4,61 (10)
B × J	4,57 (9)	7,02 (9)	6,4 (9)	3,79 (8)	4,04 (9)
(B × C) × J	4,69 (14)	7,62 (14)	7,02 (13)	4,80 (13)	5,27 (13)

Zahlen in Klammern: Anzahl der Pflanzen.

eine Pflanze der Gruppe 11/16. Die Blätter bis zur Höhe von 56 cm wurden am 25.6. gemessen; die Pflanze wurde an diesem Tage in einen 12 cm-Topf umgesetzt, blieb aber weiter im 16 Std.-Tag. Der Rest der Blätter wurde am 22.8. gemessen. Die Darstellung kommt der Wirklichkeit insofern näher, als die Abszisse die wirkliche Insertionshöhe der Blätter darstellt. Es tritt eine starke Häufung kleiner Blätter in der Blütenregion auf, was im Zusammenwirken mit der starken Verzweigung in dieser Region den kolbigen Eindruck des Blütenstandes beim Weibchen hervorruft. Der anfängliche Verlauf der Kurven ist der gleiche wie bei den 11/24 Pflanzen. 16 Std.-Tag wirkt gegenüber vorangehendem 11 Std.-Tag also ebenso wie Dauerlicht.

Auf die weitere Auswertung der Gruppen mit 16 Std.-Tag kann verzichtet werden. Grundsätzlich sind die Ergebnisse die gleichen wie bei den anderen Gruppen, doch variieren die Pflanzen auch innerhalb der Gruppen insofern sehr stark, als bei verschiedenen Pflanzen die größten Blätter nicht mehr an entsprechenden Knoten auftreten. Im einzelnen entsprechen die Gruppen 11/16=11/24; 16/16=11/11; 24/16=24/11; 16/24=11/24; 16/11=24/11. Die Klassifizierung der Gruppen 16/16 und 24/16 ist nicht ganz sicher, da der



Abb. 3. A. *Habitus einer breitblättrigen Pflanze nach der Verjüngung.*
 B. *Blätter derselben Pflanze, die Zahlen geben die natürliche Reihenfolge der Blätter an.*
 C. *Blätter einer verjüngten, schmalblättrigen Pflanze.*

Tod der Pflanzen nicht abgewartet wurde. Es besteht eine sehr geringe Wahrscheinlichkeit, daß sich diese Pflanzen doch noch verjüngt hätten. Wenn dies nicht der Fall ist, so würde sich 16 Std.-Tag gegenüber vorangehendem 11 Std.-Tag als Langtag, gegenüber vorangehendem 24 Std.-Tag als Kurztag auswirken.

Eine zweite Verjüngung (einen dritten Lebenscyclus) machte eine Pflanze der Gruppe 11/24 durch. Diese war am 25.6. nach dem Umtopfen in 12 Std.-Tag gebracht und am 25.7. wieder in Dauerlicht übertragen worden.

In einem weiteren Versuch wurde untersucht, ob sich auch Pflanzen anderer photoperiodischer Typen durch Verlängerung des Tages nach vorheriger Aufzucht und Blüte im Kurztag verjüngen lassen. Die bisher beschriebene Rasse Pelozella (P) hat eine obere kritische Tageslänge zwischen 16 und 20 Stunden, ähnlich die Rasse Carmagnola (C); ihre obere kritische Tageslänge liegt etwas über der der Rasse Pelozella. Außerdem wurden untersucht: ein indischer Hanf (J) mit oberer kritischer Tageslänge von unter 12 Stunden, und die einheimische Sorte Schurig (F), die nur quantitativen Kurztagscharakter besitzt, praktisch nahezu tagneutral ist. Ihr entspricht die monözische Sorte Bernburger Einhäusiger (B). Dazu kamen einige F_1 dieser Herkünfte, die im photoperiodischen Verhalten nahezu dem Elter mit der weniger

ausgeprägten Kurztagsreaktion gleichen. Die Pflanzen wurden im 11,5 Std.-Tag angezogen und jedes ♀ 6 Tage nach Beginn der Blüte in 24 Std.-Tag übertragen. Nach weiteren 4 Wochen wurden die Blätter gemessen. Die Pflanzen der indischen Sorte blühten sehr viel später als alle anderen Herkünfte und wurden daher nicht ausgewertet. Das Ergebnis war eindeutig; sämtliche Pflanzen wurden verjüngt. Allerdings bildeten nur C und P keine Blüten mehr aus, alle anderen Gruppen blühten kontinuierlich weiter.

Die Pflanzen dieses Versuchs besaßen 2 verschiedene Blattformen. Die indische Sorte enthält dominante Gene für Schmalblättrigkeit, alle Pflanzen mit indischem Elter waren also schmalblättrig. Die Tab. 1 zeigt die Blattformwerte beider Gipfel; die Maximalwerte (Blühbeginn) der breitblättrigen Formen fehlen zum großen Teil, da die Blätter z.Zt. der Messung bereits abgeworfen waren. Dagegen lassen sich die Minimalwerte gut vergleichen. Die Mittelwerte der Minima bei den Pflanzen mit den Genen für Schmalblättrigkeit unterscheiden sich eindeutig von denen der Pflanzen ohne diese Gene, die Variationskurven beider Gruppen überschneiden sich nicht.

Die Abb. 3 a zeigt den Habitus einer verjüngten Pflanze aus $P \times B$, Abb. 3 b die Blätter derselben Pflanze, Abb. 3 c die Blätter einer Pflanze aus $J \times B$.

Diskussion

Die Besprechung der Ergebnisse soll von der Tageslängenabhängigkeit der Blattgröße ausgehen. Diese Abhängigkeit ist keine neue Erkenntnis (z.B. Bünning u. Konder 1954, und Arney 1956). Beim Hanf gilt sie vor und während der Blüte und zwar derart, dass für eine entsprechende Grössenzunahme der Blätter vor der Blüte nur ein erheblich kürzerer Tag erforderlich ist, als während der Blühperiode. Dies folgt daraus, dass eine Pflanze im gleichen Kurztag vor der Blüte fortlaufend grössere, nach Beginn der Blüte fortlaufend kleinere Blätter ausbildet. Offenbar konkurrieren nach Beginn der Blüte reproduktive und vegetative Organe um das von den älteren Blättern gelieferte Baumaterial. Wird der Tag nach Beginn der Blüte um soviel verlängert, dass der Bedarf der bevorzugten reproduktiven Organe aus der Mehrleistung gedeckt ist, so wird der Erschöpfungsverfall solange hinausgezögert, bis der gestiegene Bedarf der Blütenanlagen die Mehrleistung überschreitet. Wirkt der Langtag zudem hemmend auf die Blütenausbildung aus (bei qualitativen Kurztagstypen), so kann die Pflanze einen zweiten Entwicklungscyclus oder deren mehrere erleben. Vielleicht ist die Blattgröße einer Hanfpflanze der geeignete Anzeiger ihres physiologischen Alters, d.h. ihrer Lebenserwartung (v. Denffer, 1953), vorausgesetzt, daß die Bedingungen, unter denen die letzten Blätter ausgebildet wurden, auch weiterhin für die Pflanze gelten. Bei

manchen Arten soll die Blattform ein Maß des physiologischen Alters sein. Beim Hanf gilt dies sicher nicht. Die Blattform zweier Pflanzen am Ende der Blühperiode ist gleich, unabhängig davon, ob sie bald absterben oder einen neuen Lebenscyclus durchmachen. Es besteht aber die Möglichkeit, daß die Blattform selbst von der Blattgröße abhängig ist, und zwar derart, daß bei Zunahme der Blattgröße die neuen Blätter schmaler determiniert werden, bei Abnahme der Blattgröße breiter. Dies stimmt gut überein mit der Beobachtung Njokus' (1956), daß bei *Ipomoea* die Blattlappung nur dann zunimmt, wenn alte Blätter zugegen sind, daß aber die neuen Blätter weniger gelappt werden, wenn die alten Blätter entfernt sind. Die Größe der alten Blätter ist nur für die neu anzulegenden Blätter von Bedeutung. Schon formdeterminierte Blätter wachsen erst noch in der festgelegten Form aus. So erklärt sich z.B., daß der Blattquotient seinen größten Wert erst erreicht bei Blättern, die einige Knoten über den größten Blättern vor der Blüte ausgebildet werden. Eine Stütze dieser Ansicht, daß die Blattform schon sehr frühzeitig determiniert ist, gibt die häufig gemachte Beobachtung, daß bei Pflanzen, die schon bei Blühbeginn in längeren Tag übertragen werden, der Blattquotient nicht den minimalen Wert erreicht wie bei späterer Übertragung, sondern nur wenig abfällt und dann wieder ansteigt.

Der Versuch, die Veränderungen der Blatteigenschaften zu Vorgängen, die die Blüte auslösen, in Beziehung zu setzen, gelingt nicht. Es ist z.B. nicht einzusehen, warum bei qualitativen Kurztagstypen, wie dem Pelozellahanf, die Blüte nach der Verjüngung unterbrochen wird, bei den nur quantitativ reagierenden Typen aber nicht, obwohl sie sich in der Blattform nicht und in der Blattgröße nur wenig unterscheiden und dies wahrscheinlich aufgrund des Fortfalls der Konkurrenz zwischen Blüten- und Blattanlagen bei ersteren. Nur ein bekanntes Phänomen dürfte in diesen Zusammenhang einzuordnen sein: Pflanzen, die zunächst im Langtag aufgewachsen waren, blühen erheblich reicher als kleinere, die nur Kurztag erhalten hatten. Die größeren Blätter könnten für reichlicheren Nachschub an Baumaterial für die Blüten sorgen, damit könnten sie durchaus als Gradmesser für die Stärke des Blühimpulses dienen. Dies ist nun von außerordentlicher Bedeutung für die Geschlechtsausprägung des Hanfes. Monözische Hanfsorten bilden nur im sommerlichen Langtag, wenn sie groß und reich beblättert sind, männliche Blüten aus. Im winterlichen Kurztag bleiben sie dagegen rein weiblich (v. Sengbusch 1952). Monözisten sind proterandrisch, sie bilden zunächst männliche und erst später weibliche Blüten aus, entsprechend der Abnahme der Blattgröße. Auch die Feststellung, daß monözische Hanfpflanzen nach vorheriger Anzucht im Langtag und anschließender Übertragung in Kurztag stärker männlich blühen (Borthwick u. Scully, 1954), ist so zu erklären. Wir haben photoperiodisch qualitativ reagierende monözische Hanfpflanzen mit

Tab. 2. *Geschlechtsausprägung und vegetative Eigenschaften einer monözischen, qualitativ reagierenden Kurztagspflanze (Ende des 1. und 2. Cyclus).*

Blatt	Blüte neben der Blattachsel	Blattindex ¹	Blattquotient	Blattprodukt/10
13—16	je 1 ♂	—	—	—
17	1 ♂	—	—	—
18	2 ♂	—	—	—
19	2 Früchte	—	—	—
20	2 ♂	3	5,1	46
21	2 ♂	3	Bl. verkrüppelt	—
22	2 ♂	4	2,7	130
23	2 ♂	1	Bl. verkrüppelt	—
24	1 ♂	1	Bl. verkrüppelt	—
25	1 ♂	5	2,7	156
26	2 ♂	22,5	3,5	138
27	—	30	4,5	105
28	—	40	4,6	94
29	—	30	4,4	87
30	—	52,5	5,0	79
31	1 ♀ (3 Narb.)	42	5,3	70
32	2 ♂	52,5	5,3	79
33	2 ♂	62,5	5,4	79
34	2 ♂	52,5	5,4	65
35	1 ♂	55	5,5	61
36	1 ♂	55	6,1	47
37	1 ♂	57,5	6,7	26
38	1 ♂	60	6,4	19
39	1 Frucht	65	7,75	12
40	1 ♀	Bl. nicht meßbar klein	—	—
41	1 Frucht	—	—	—

¹ Nach Heslop-Harrison.

Dauerlicht verjüngt und dann wieder in Kurztag übertragen. Die männlichen Blüten traten in beiden „Generationen“ vor den weiblichen und oberhalb der größeren Blätter auf; ein Beispiel zeigt die Tab. 2.

Von Sengbusch fand in seinem Hanfmaterial Habitusformen, die er als „Wuchsmutante locker“ bezeichnet. Bei diesen Typen handelt es sich offenbar um verjüngte Pflanzen. Sie entstehen vor allem bei sehr zeitiger Aussaat im Frühjahr. Im vorsommerlichen Kurztag beginnen sie schon als sehr kleine Pflanzen zu blühen, wachsen dann aber bei steigender Tageslänge weiter und erhalten auf diese Weise den aufgelockerten Blütenstand. Ob allerdings verjüngte oder lockere Pflanzen mit allen weiblichen oder monözischen Pflanzen vom männlichen Habitus, die Hoffman (1947/1952) beschreibt, identisch sind, wie v. Sengbusch vermutet, können wir nicht entscheiden.

Zusammenfassung

Form und Größe der Blätter des Hanfes ändern sich im Laufe der Entwicklung gesetzmäßig. Die Blattgröße wird von der Tageslänge beeinflusst, die

Blattform scheint von der Größe der älteren Blätter abzuhängen. Im Kurz- und Langtag werden aufeinander folgende Blätter von den Kotyledonen an immer größer, ihre Blättchen immer schmaler. Im Langtag wird ein Endwert erreicht, im Kurztag beginnen die Pflanzen zu blühen, ihre neuen Blätter werden immer kleiner, aber relativ breiter. Überträgt man eine blühende Pflanze in Langtag, so geht die relative Verbreiterung der Blätter wie im Kurztag weiter, doch werden die neuen Blätter nicht fortlaufend kleiner, sondern wieder größer. Die Pflanze „verjüngt“ sich. Am Ende der Verjüngung stehen Blätter, die sich von normalen Primärblättern nur dadurch unterscheiden, daß sie größer sind. Von diesem Stadium an beginnt sowohl im Langtag als auch im Kurztag ein neuer Lebenscyclus mit relativer Verschmälerung und Vergrößerung der Blätter bis zur Blüte und folgender Verkleinerung und relativer Verbreiterung der Blätter bis zum Tod. Eine zweite Verjüngung wird möglich, wenn der verjüngten Pflanze bis zur zweiten Blüte Kurztag und anschließend Langtag geboten wird. Als Langtag wirkt jede ausreichende Verlängerung der Lichtzeit, z.B. 16 Stunden gegenüber $10\frac{3}{4}$ Std. und 24 Std. gegenüber 16 Std.

Die Blattgröße nach Blühbeginn ist ein Maß des physiologischen Alters der Pflanze (definiert als Lebenserwartung).

Mit der Blattgröße scheint bei Monözisten die Ausprägung des Geschlechts derart gekoppelt zu sein, daß in Sproßzonen oberhalb großer Blätter männliche, oberhalb kleiner Blätter weibliche Blüten ausgebildet werden.

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Obligate Phototrophy in *Chlamydomonas eugametos*

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Introduction

Obligate phototrophy — the inability to grow in the absence of photosynthesis — is fairly common among microorganisms. Certain blue-green algae (Fogg 1953, Kratz and Myers 1955), diatoms (J. C. Lewin: 1953), green algae (J. C. Lewin 1950, R. A. Lewin 1954, Finkle *et al.* 1950, Lucksch 1932) and photosynthetic bacteria (Woods and Lascelles 1954) exhibit this dependence. Strict autotrophy may place such organisms at a selective disadvantage in habitats rich in organic matter, however, in habitats low in organic matter, curtailment of the energy consuming reactions of growth may bring about conservation of endogenous reserves and extend the period through which the cell can survive darkness. In certain species of *Chlamydomonas* growth ceases rapidly after removal of cells from the light. Nearly all cells become motile and strongly phototactic at this time. Continuous motility ensues until all reserves and a large part of the protoplast have been utilized. This diversion of available reserves into a cellular mechanism which could achieve relocation of the cell in the light may have survival value.

J. C. Lewin (1950) made an extensive search for organic materials which would serve as substrates for the growth of *Chlamydomonas moewusii* in the dark. She tested filtrates from cultures grown in the light, over 60 organic materials, and cell extracts and homogenates. She found that no autoinhibitors were produced in the dark. The cell membrane apparently was perme-

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able to organic acids which can serve as substrates for the growth of other algae in darkness but which do not support growth of this species in the dark. She concluded that CO_2 is the only carbon source which this organism can utilize.

During a study of induced mutants of *Chlamydomonas eugametos* Moewus — a species possibly inter-fertile with *C. moewusii* — it was found by Wetherell and Krauss (1957) that wild type cells could not grow in the dark on any of several complex organic media. Attempts to induce mutants which could overcome this metabolic block in darkness were unsuccessful although over 2 million cells were irradiated with X rays and given sufficient time to overcome phenomic lag before transfer to darkness on organic media. The following experiments characterise obligate phototrophy in this species more fully and test a number of hypotheses concerning its nature.

Materials and Methods

Two basal media were used interchangeably throughout these studies. The principal difference was the method of supplying trace elements. The first medium that of Myers (Sorokin and Myers, 1957) (KNO_3 , 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0; KH_2PO_4 , 1.25 grams per liter). Relatively high levels of trace elements were chelated by the addition of 0.5 grams of ethylenediaminetetraacetic acid per liter of medium. The second contained less $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.25 grams per liter) and used non-chelated trace elements supplied at the levels recommended by Allen and Arnon (1955). Iron was supplied in the latter case at 4 ppm. as the ethylenediaminetetraacetate. The pH of all media was adjusted to 6.8. Myers' medium had the advantage that insoluble magnesium phosphate, formed by heating, was redissolved in the presence of the high level of chelator, but the high organic nitrogen level of this medium was undesirable during studies of nitrogen metabolism. Growth rates and total growth of *C. eugametos* in both media were the same.

The constituents, concentrations, and preparation of the organic acid mixture, sugar mixture, vitamin mixture, nucleic acid hydrolysate, and complete medium are as given by Wetherell and Krauss (1957). The cell-homogenate was prepared according to the technique of French and Milner (1951), and was passed through a Seitz filter to free it of bacteria.

All experiments were carried out at 25°C. "Cool white" fluorescent lights were used at intensities given with each experiment. Aerated cultures received a mixture of 4 per cent CO_2 in air. Cotton stoppered 18×150 mm. Pyrex testtubes with 2 mm. Pyrex tubing inserted through the stopper and for aeration, were used for all growth tests. Periodic readings of optical density at 560 mμ were made by placing culture tubes in a B & L Spectronic 20 spectrophotometer. Culture tubes used in this way were calibrated immediately after inoculation to correct for differences in diameter.

In order to obtain suspensions in which the cells were in the same stage of development, cultures were given two cycles of 15 to 18 hours of light followed by 12 hours of darkness. At the end of the next light period the accumulated mother cells tend to clump and settle to the bottom of the vessel. Cells in other stages were removed

by repeated, gentle centrifugation. After another dark period a uniform suspension of very small daughter cells were obtained.

Conventional manometry was used with the exception of the substitution of diethanolamine, saturated with 2.5 per cent CO_2 according to the directions of Krebs (1951), for the usual KOH solution. Preliminary comparisons showed no differences in results due to choice of CO_2 absorbent. The direct two-vessel method (Umbreit *et al.* 1951) was used to determine CO_2 evolution during respiration.

The exact time of mitosis during the life cycle was determined by the use of the staining procedures of Schaechter and De Lamater (1955).

Chlorophyll was measured as optical density of acetone extracts at a wavelength of 630 m μ . A five-minute extraction of chlorophyll was made at room temperature with 80 per cent acetone. The insoluble organic nitrogen fraction was determined directly by the digestion and analysis of the residue from cells extracted at 75°C, once with 1 per cent acetic acid and three times with distilled water.

Nitrate uptake was measured by a modified phenoldisulfonic acid method in which the reagent was added directly to the culture medium. Reagents and procedures were otherwise the same as those of Johnson and Ulrich (1950). Ammonium uptake was measured by nesslerization of the culture medium and organic nitrogen by slight modifications of the method Umbreit, *et al.* (1951). The modifications consist of digestion at 280–300°C and omission of hydrogen peroxide.

Pure cultures of the "male" strain of *Chlamydomonas eugametos* Moewus obtained from Dr. Harold Bold were used through this study.

Results

Characterization of obligate phototrophy. — The extent of inhibition of growth in the absence of light was shown in several preliminary tests (Wetherell and Krauss 1957). When cultures were permitted to reach a rapid growth rate under normal culture conditions in light, in either inorganic or complex organic media, and were then transferred to darkness, growth measured as optical density ceased abruptly. After two days a gradual decrease was noted. Microscopic examination of the cells during this dark period showed disappearance of granular reserve materials followed by gradual contraction of the protoplast until, at the end of eight days, the protoplast occupied only the anterior quarter of the cell leaving an empty, translucent lumen within the remaining, visibly unchanged, cell wall. The cells were assumed to be viable at this time because nearly all were actively motile.

A detailed comparison of the rate of change of dry weight and optical density during photosynthesis and in the transition to dark-metabolism can be obtained from figure one. From the seventh to the tenth hour the rate of loss of dry weight is very high. The dissolution of cell-walls during daughter cell discharge may account for part of this loss.

When a suspension of uniformly young daughter cells was spread on the

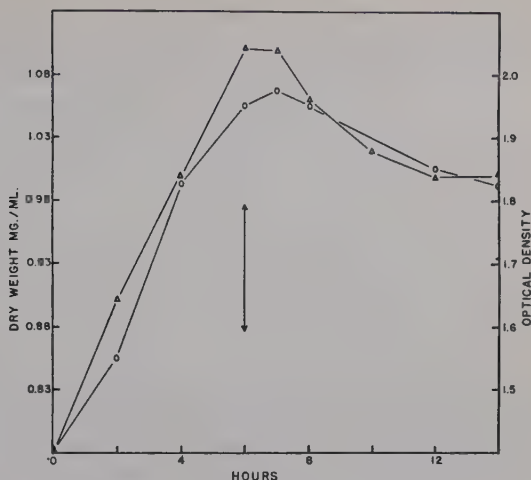


Figure 1. Dry weight (\triangle) and optical density (\circ) of cells of *Chlamydomonas eugametos* cultured in light for six hours and then transferred to darkness (arrow). 1400 f.c. illuminance; aerated with 4 per cent CO_2 in air.

surface of agar containing complete medium and the cultures placed in darkness, no cell division took place during the 72 hour test period in contrast to small colony formation in lighted cultures. If dark-treated cultures were replaced in light at this time cell division occurred and normal growth resumed.

The light requirement. — Three tests were made to determine the nature and extent of the light requirement. In order to test for a photochemical requirement other than photosynthesis, cells were placed on the surfaces of agar containing the complex organic medium and agar with inorganic medium and incubated in a lighted chamber (500 f.c.) made deficient in CO_2 by a large surface area of KOH solution. After five days, control cultures with CO_2 at the concentration normally found in air, showed 200–300 cells per colony in contrast to a maximum of four cells per colony (50 per cent of cells remained undivided) in CO_2 -deficient cultures. A return of CO_2 -deficient cultures to CO_2 -sufficient conditions resulted in normal colony development. Inorganic and organic media gave similar results.

The possibility that trace amounts of CO_2 are required for growth made it desirable to test the light requirement in a different way. It will be shown below that certain organic compounds increase the rate of oxygen uptake by *C. eugametos*; however, these do not support growth in the absence of light. If the respiration of these compounds provided ample energy and metabolites, it might only remain necessary to supply light at very low intensities to trigger their utilization for growth. One might at least expect the oxidizable compounds to contribute to growth processes under conditions where light level is too low to maintain a high growth rate. To test this, the relationship between light intensity and growth was determined and tests of organic compounds were made at a light intensity just sufficient to maintain detectable growth. The basal medium in these tests contained an ammonium source and

Table 1. Test for growth stimulation by common substrates supplied to cultures of *Chlamydomonas eugametos* growing at 45 f.c. illuminance. Readings after 4 days of culture. Initial optical density was 0.04. All media include the inorganic basal components.

Medium	Concentration	Optical density at 560 m μ .
Basal.....	—	0.45
Casein Hydrolysate	1.0 g./l.	0.35
Glucose	10^{-2} M	0.35
Na-acetate	10^{-2} M	0.34
Complete	—	0.37

trace amounts of water-soluble vitamins in addition to the usual inorganic constituents. Cultures were aerated with 4 per cent CO₄ in air. Growth was found to be approximately proportional to light intensity over the range from 45 to 950 foot candles. Growth occurred at a steady but very low rate at the lowest light intensity.

Forty-five foot candles of illuminance was chosen and growth on basal medium at this level was compared with that on basal medium supplemented individually with: casein hydrolysate, complex organic medium, glucose, and acetate. Optical densities of cultures at the end of four days are given in Table 1.

An investigation was then made of the stage or stages in the life cycle which require light for their completion. Light requirement for a single developmental step might prevent normal growth just as effectively as curtailing the source of metabolic energy. The life cycle of *C. eugametos* can be divided into several stages on the basis of physiological, morphological, and cytological changes. A newly formed daughter cell placed under normal culture conditions (25°C, aerated, ca. 1400 f.c. illuminance) undergoes a more or less steady increase in volume and weight during the first 12 to 15 hours. Nuclear stains show that mitosis occurs between 12 and 15 hours giving rise successively to eight nuclei. Cytoplasmic cleavage simultaneously delimits eight equal-sized daughter cells. During the period from 15 to 20 hours, these cells increase in size and differentiation until at approximately 20 hours, they are released from the mother cell membrane. Cells placed in darkness at the time of mitosis, discharge normal daughter cells but these undergo no further detectable development until they receive light. The development of cells placed in darkness before mitosis depends upon the length of the prior photosynthetic period. Table 2 shows these relationships. Mitosis and formation of daughter cells does not require light if the pre-mitotic cell has received more than six hours of normal culture conditions. However, the number of daughter cells formed from each mother cell varies with the length of the light period up to 12 hours. Although new daughter cells receiving

Table 2. *The relationship between the number of hours of light received by newly formed daughter cells of a synchronized culture of Chlamydomonas eugametos Moewus and the completion of cell division after transfer to darkness. Numbers in parentheses give the number of daughter cells produced by each mother cell.*

Hours of light before transfer to darkness	Time of visual checks in hours after beginning of experiment				
	13	15.5	19	20.5	24
0	No division	—	No division	—	No division
3	No division	—	No division	—	No division
6	No division	—	No division	—	No division
9	35 % divided (2,4)	50 % divided (4)	No further division	All daughter cells discharged (4)	—
12	85 % divided (4,8)	—	20 % discharged	—	All daughter cells discharged (4,8)
15	—	90 % divided (8)	80 % discharged	All daughter cells discharged (8)	—

six hours of light seemed to have adequate insoluble carbohydrate reserves, no further visible development took place in the absence of light.

Nutritional studies. — Prior to detailed metabolic studies the alga's response to exogenous organic materials was tested more completely. A complex medium consisting of sugars, amino acids, organic acids, nucleic acid hydrolysate, and vitamins as well as media containing individual sugars, amino acids, or organic acids failed to support growth in darkness. Seitz-filtered, freshly-prepared homogenate of cells grown in the light was added at several dilutions to media supplemented with ammonium, vitamins, and 5×10^{-3} M sodium acetate. Growth in light was unaltered by these additives. In the absence of light, over a five day period, all treatments showed a decrease in optical density indicating that no growth had occurred.

The addition of adenosine triphosphate (ATP) to cultures supplemented with ammonium, water-soluble vitamins, 5×10^{-3} M glucose or succinate, also failed to support growth in darkness. However, all cultures containing added ATP were darker green than controls after seven days of culture in darkness indicating that ATP or a breakdown product had entered the cell.

Manometric studies of substrate oxidation. — J. C. Lewin (1950) reported that acetate, pyruvate, and succinate are readily oxidized by *C. Moewusii* in darkness. Acetate will support dark growth of a number of phytoflagellates which are incapable of utilizing glucose. Data showing the effect of acetate on the rate of O_2 uptake are given in Table 3. R. A. Lewin (1954) studied the effect of acetate on the respiration rate of *Chlamydomonas dysosmos*. He found that cells which had been grown in liquid culture, (aeration, 450 f.c. illuminance) and had not been subjected to a period of starvation after growth in light, showed no detectable stimulation of oxygen uptake on the addition of any substrate. However, after aeration for three days in darkness, the endogenous rate was decreased and stimulation of respiration by acetate

Table 3. *The effect of dark-induced starvation on the endogenous and acetate respiration rates of Chlamydomonas eugametos Moewus.*

Hours of Darkness	Average rates of O ₂ uptake in μ l. O ₂ /hour/cmm of cells	
	Endogenous	1/100 M Acetate
0	3.4	2.1
24	2.3	2.1
48	1.8	2.3
102	0.56	0.8

was observed. Cells grown seven days on a solid agar medium at 220 f.c. showed stimulation by acetate even when not subjected to previous starvation in darkness. Cells cultured under the latter conditions are probably as starved physiologically as the dark-treated cells from liquid culture because of mutual shading, low light intensity, and poor gaseous exchange. The very low endogenous respiration rate in these experiments (0.5—0.8 μ l./hour/cmm. of cells) further suggests that the cells were in poor condition. Therefore the reported differences in the degree of response due to culture conditions are probably not of metabolic significance. In the present study respiration rate in the presence of acetate exceeds the endogenous rate after 24 hours of starvation but at no time exceeds the endogenous rate of non-starved cells.

Respiration, in the presence of a number of the compounds and mixtures used in the growth studies, is shown in Table 4. Yeast extract was later added to test for effects of vitamins and growth factors not provided with the substrates. However, this caused no significant changes. Vitamin-free casein hydrolysate and organic acid mixture (pyruvic, succinic, fumaric, citric, acetic, glycolic, gluconic, lactic, and malic acids) caused a 24 per cent and 37 per cent increase, respectively, in O₂ uptake of these 30-hour starved cells. However, neither complex will support growth in the absence of light. Ammonium chloride was used to indicate the extent of depletion of reserve. This is a useful test because there is little stimulation of respiration when ammonium is added to starved cells. (Contrast with table 5).

Comparison of the rate of CO₂ evolution with the rate of O₂ uptake of cells in inorganic medium indicated that O₂ uptake was a valid measure of respiration.

Nitrogen metabolism. — Growth in the sense of increase in protoplasm results largely from the conversion of soluble, nitrogenous organic compounds into protein and nucleic acid macromolecules. Endogenous respiration of obligate phototrophs may not be capable of producing sufficient energy or certain key intermediates required for these condensations.

Table 4. *The effect of some common substrates on the respiration rate of 30-hour dark-starved cells of Chlamydomonas eugametos Moewus.* Data are given a μ l. O₂ uptake/hour/cmm. of cells. The inorganic medium was a constituent of all media.

Inorganic medium	2.0	Organic acid Mixture	2.7
NH ₄ Cl	2.1	"Sugar" mixture	2.1
1/100 M Glucose	2.1	Nucleic acid hydrolysate ...	2.0
1/100 M Glycine	2.1	Casein hydrolysate	2.5
Vitamin mixture	2.0	Complete medium	2.7

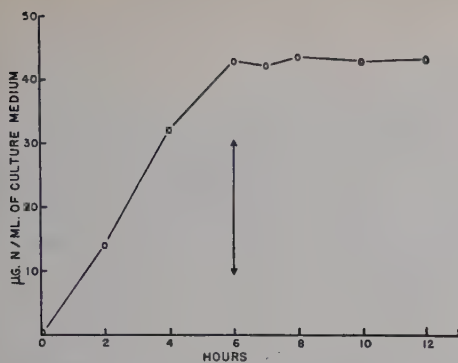


Fig. 2.

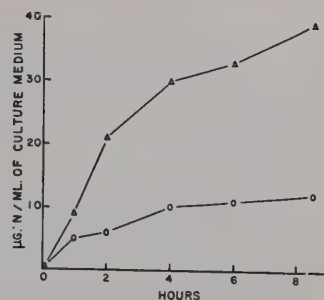


Fig. 3.

Figure 2. Uptake of nitrate from the culture medium by N-sufficient cells of *Chlamydomonas eugametos* in light and darkness. Arrow indicates time of transfer to darkness. 100 µg nitrate-N per ml; total organic-N of cells at zero hour, 112 µg per ml; 1400 f.c. illuminance; aerated with 4 per cent CO₂ in air.

Figure 3. Uptake of nitrogen from the medium by nitrogen-deficient cells of *Chlamydomonas eugametos* cultured in darkness on nitrate — (O) and ammonium — (Δ) containing media. 150 µg ammonium-N per ml.; 100 µg nitrate-N per ml.; insoluble organic-N at zero hour, 80 µg per ml.; aerated with 4 per cent CO₂ in air.

Considering the rate of endogenous respiration (Table 3) and the rate of decrease of dry weight (Figure 1) in the dark, one might expect some N-assimilation by darkened cells. However nitrate assimilation in N-sufficient cells ceased abruptly upon transfer to darkness (Figure 2). N-sufficient cells of *Chlorella pyrenoidosa*, a facultative heterotroph, continue to assimilate nitrate-N at a high rate for more than three hours after transfer to darkness (Schmidt 1957).

Eight hour N-deficient cells placed in darkness were capable of absorbing small amounts of nitrate and considerable quantities of ammonium nitrogen from the medium (Figure 3). The endogenous respiration rate of cells cultured for 12 hours, in the light, in N-free medium, was sharply increased when either KNO₃ or NH₄Cl were added (Table 5). The differences between rates on KNO₃ and NH₄Cl do not necessarily reflect differences in rate of N-metabolism. Syrett (1955) has shown the R.Q. for ammonium assimilation to approach 1.0 while for nitrate assimilation it may exceed 2.0. Therefore differences in rate of O₂ uptake may be misleading. However, studies of uptake of nitrate and ammonium from the culture medium have

Table 5. Respiration rates of 12 hour nitrogen-deficient cells of *Chlamydomonas eugametos* Moevus with and without added nitrogen sources. Data in µl. O₂ uptake/hour/cmm. of cells.

Medium	Concentration	Rate
Basal.....	—	2.6
Basal + KNO ₃	139 µg.N/ml.	3.6
Basal + NH ₄ Cl.....	139 µg.N/ml.	6.2

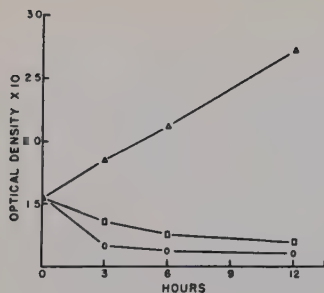


Figure 4. Chlorophyll synthesis in cells of *Chlamydomonas eugametos* cultured in light (Δ), in darkness on inorganic media (\square), and in darkness on media containing 0.05 per cent glycine and acetate (\circ). 1400 f.c. illuminance; aerated with 4 per cent CO_2 in air. Data given as absorbancy of acetone extracts at 630 m μ .

shown (Figure 3) that rate of uptake of nitrate is also lower than that of ammonium during the first two hours of the experiment. (The period during which respiration rates were determined.) These data establish the metabolic uptake of nitrate and ammonium from the medium.

The extent of incorporation of nitrogen into insoluble constituents was next determined as a means of estimating synthesis of N-containing macromolecules. Net synthesis of chlorophyll by N-sufficient cells in ammonium containing media stops abruptly when cells are removed from light (Figure 4). Other experiments (not shown) included ATP, casein hydrolysate, glycine, and acetate individually and in combinations in the culture medium without demonstrating a stimulation of chlorophyll synthesis.

The time-course of uptake and incorporation of nitrogen into the total insoluble fraction by N-deficient cells in ammonium containing medium, in the dark, is given in Figure 5. Different levels of ammonium were used to determine if uptake would exceed incorporation. It was felt that the drain placed upon energy reserves by reactions incorporating excesses of ammonium into basic amino acids and amides (Syrett 1954) might reduce energy available to normal synthetic pathways. It was found however that uptake closely paralleled incorporation with the exception that uptake exceeded incorporation by approximately 35 per cent at the three highest levels. Net synthesis of insoluble nitrogenous compounds ceased 10 hours after cells were removed from the light (eight hours after addition of ammonium) and a rapid dissolution reduced the insoluble fraction nearly to the starting level by the 24th hour. Microscopic examination of the cells at the 24th hour showed that the cells were still rich in granular reserve carbohydrate and thus not substrate deficient. All cells ceased incorporation at the 10th hour independent of the amount of incorporation at that time. A repetition of this experiment gave identical results with the exception that breakdown of the insoluble fraction occurred in the 9th hour after addition of the nitrogen source. In another experiment, 1×10^{-2} M glucose, 5×10^{-3} M acetate and 5×10^{-3} M pyruvate were added individually to cultures containing N-starved

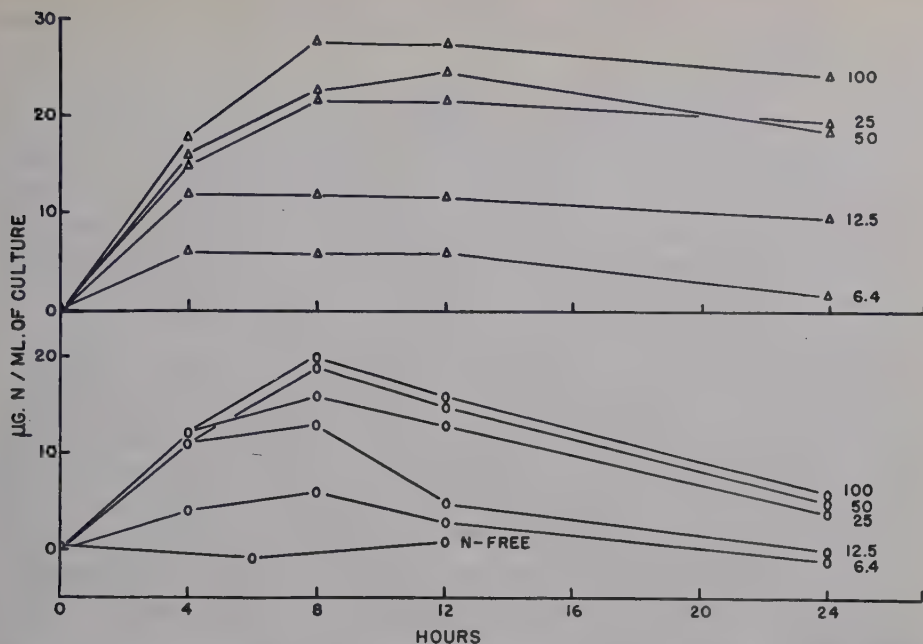


Figure 5. Uptake of ammonium-N from the medium (Δ) and incorporation of this nitrogen into the insoluble organic-N fraction of the cells (\circ) by N-deficient cells of *Chlamydomonas eugametos* cultured in darkness at different levels of ammonium-N. The level of ammonium-N is given at the end of each curve. Insoluble organic-N at zero hour was 42 µg per ml. in each culture; aerated with 4 per cent CO_2 in air.

cells and 25 µg./ml. of ammonium-N. Incorporation of insoluble-N followed the same course as the curve for 25 µg./ml. in Figure 5. These compounds contributed nothing toward incorporation or retention of the insoluble-N fraction. The insoluble-N of cells placed in N-free medium, did not undergo dissolution at the eight hour of the experiment (Fig. 5).

Discussion

A number of hypotheses must be considered in an attempt to understand the nature of obligate phototrophy in *C. eugametos*.

1. Light may be required to provide a state of high energy availability either directly through photosynthetic phosphorylation (sensu Arnon 1956) or indirectly by providing photosynthate to enhance respiratory phosphorylation.

2. A critical photochemical reaction apart from photosynthesis may exist.

3. Autoinhibitors may be produced in the dark as the result of dissociation of light dependent reactions.

4. One or more building-blocks essential to the synthetic reactions of growth may be formed exclusively during photosynthesis.

5. The inhibition of photosynthesis may create a physico-chemical state which is unfavorable for one or more essential synthetic reactions, yet which permits or actually enhances certain other reactions. Shifts in intracellular pH or oxidation-reduction potential are envisioned as the controlling factors. The light-induced inhibition of pyruvate decarboxylation proposed by Calvin (1955) may serve as an example of this type of control.

Dark metabolism of *C. eugametos* is by no means quiescent. The endogenous respiration rate is higher than that of green algae with similar growth rates (Meyers 1949, Nihei *et al.* 1954). Darkened cells maintain uninterrupted motility for as long as eight days. This process is most certainly dependent upon energy-yielding metabolism. The carbohydrate reserve, which shows the staining characteristics of amylopectin, is completely utilized during this period and respiration rates seem to parallel this utilization (Table 3). Mitosis, cleavage, and release of daughter cells proceeds in darkness (Table 2).

The importance of a direct photochemical phosphorylation as an energy source is minimized by the demonstration that cells placed in the light in a CO₂-free atmosphere did not grow. Although the possibility remains that energy which drives these processes is not adequate for protein and nucleic acid synthesis, this evidence of an active metabolism in the absence of light detracts considerably from hypothesis number one.

The importance of a photochemical reaction other than photosynthesis (hypothesis number two) is minimized by the data presented above in the section on light requirement. A wide variety of potential substrates contribute little or nothing to development in the light under conditions restricting photosynthesis. Cell division apparently has no special light requirement.

The third hypotheses seemed doubtful in view of the work of J. C. Lewin (1950) who found no photostable, toxic substances produced by darkened cells. Furthermore in the present study, cultures returned to the light after extended periods of darkness grew normally.

With regard to hypothesis number four, comprehensive attempts made in this study and in the work of J. C. Lewin (1950) to find substrates or cofactors which would permit growth in the absence of light, show that no measurable changes occurred when amino acids, organic acids, sugars, vitamins, etc. were added to the culture media. There can be little doubt that many of these compounds are present naturally in cells of *C. eugametos*. The extensive negative evidence from feeding experiments is not decisive, however, because

of the uncertainty of penetration and the countless intermediates not as yet tested.

It has been suggested (*e.g.* Steward and Thompson 1950) that protein synthesis in chlorophyllous cells can proceed by different pathways than in non-green cells. Data presented by Calvin and coworkers (1955), from studies of photosynthesis in the presence of $C^{14}O_2$, show that heavy labeling of protein occurs with only slight labeling of Krebs-cycle acids. This may be interpreted as direct utilization of photosynthetic intermediates for protein synthesis. No mechanism seems to have been discovered for such conversions.

Studies on the formation of insoluble, nitrogenous, organic compounds (Figure 5) indicate that this species is incapable of continued synthesis of stable proteins and nucleic acids in darkness even with high levels of endogenous reserve. Although the pattern of synthesis is suggestive of a gradual depletion of a light-formed intermediate compound, the cessation of a net synthesis at the eighth hour is independent of the total synthesis at that time. Another experiment (data not presented) gave similar results. Net synthesis ceased in the ninth hour, regardless of the ammonium level of the medium, as though independent of the total amount of available intermediates.

The alteration of intracellular environment suggested in hypothesis five implies a suppression of the energy consuming reactions of growth with either no effect upon or a stimulation of certain other systems. Actually removal of light stops protein and nucleic acid synthesis and stimulates motility in *Chlamydomonas*. It is felt that the high endogenous respiration rate of this species is largely due to metabolism supporting flagellar activity with only a minimum contribution toward the maintenance of other cellular processes. The increase in motility of darkened cultures may be the indirect result of termination of other energy consuming reactions however the stimulation of motility seems to precede other changes.

Despite the manifestations of an active metabolism in the dark, new protoplasm is not synthesized. Furthermore with the possible exception of non-assimilative oxidation of certain organic acids, these processes seem largely unaffected by the addition of common substrates, cofactors, crude extracts, and homogenates to the culture medium. The length of the period of motility is not extended by such additions, the light requirement of the first half of the life cycle cannot be significantly shortened, nor do these organic compounds increase the growth rate at low light intensities or detectably alter the rate of disappearance of the endogenous reserve in the dark. It seems particularly significant that a relatively high rate of endogenous respiration proceeds without contributing to a significant net-synthesis and extended stability of insoluble nitrogenous products. Failure of growth on complex organic media in the light in the absence of CO_2 minimizes the

role of light apart from its role in photosynthesis. Evidence seems to favor an interpretation of obligate phototrophy in which certain products of photosynthesis or a physico-chemical state dependent upon photosynthesis control both metabolism at the protein and nucleic acid synthesizing sites of the cell and the diversion of energy into the maintenance of controlled cellular motility.

Summary

Obligate phototrophy in *Chlamydomonas eugametos* has been characterized in sufficient detail to indicate the nature of the interrelationship between removal of light and cessation of growth. Experiments carried out in light under conditions restricting photosynthesis preclude the importance of non-photosynthetic photochemical reactions. No light requirement was found for the stages of development during which mitosis, formation of daughter cells and release of daughter cells take place. Extensive feeding experiments with both individual organic compounds and complex natural sources of metabolites and growth factors failed to permit detectable growth in darkness.

Manometric studies of respiration showed that endogenous respiration rate of non-starved cells could not be increased by the addition of exogenous potential substrate materials, but cells starved in excess of 24 hours showed increased rates when acetate and certain crude substrate sources were supplied. Glucose was not effective under these conditions. The increase in respiration rate under these conditions did not exceed the endogenous rate of non-starved cells. None of the substrates which increase respiration of starved cells would promote growth.

Increase in cellular nitrogen ceased abruptly when N-sufficient cells were darkened. N-deficient cells continued to incorporate N into an insoluble form for a brief period after darkening. The rate of incorporation dropped rapidly and nearly all the insoluble-N formed during this period was lost from this fraction within 24 hours.

Obligate phototrophy in this species is attributed to a shift in the physico-chemical state of the cell which results from the inhibition of photosynthesis. One or more essential synthetic reactions are inhibited by this changed environment. Inhibition of synthetic reactions is reflected in the rapid cessation of incorporation of N into insoluble forms. Flagellar activity is increased by the shift to dark-metabolism and continues at a high rate at the expense of cellular reserves for up to eight days after darkening the culture. The economy of the cell appears so altered by the transition from light to

darkness as to make all reserves available for the maintenance and control of cellular motility.

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The Effect of the Past History of Cells of *Chlorella* on Their Photosynthetic Capacity

By

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Photosynthetic capacity of cells depends on the genetic make up of the organism and external conditions. The external factors operating during a photosynthesis experiment, as well as the environmental influences to which cells are subjected prior to photosynthetic measurements, are of great importance. The internal capacity of the photosynthetic mechanism is affected by preconditioning, that is, by the past history of cells. This paper reports an investigation of the dependence of photosynthetic capacity on light intensity and temperature both during and prior to photosynthetic measurements. High-temperature algae with high metabolic activity and a broad tolerance to temperature and light intensity conditions for their growth proved to be particularly suitable in this study.

Material and Methods

The high-temperature strain, *Chlorella* 7-11-05, isolated from local waters in Texas in 1951 (Sorokin and Myers, 1953), was used in all experiments. Cells were grown in a continuous culture apparatus (Myers and Clark, 1944) under conditions permitting the maintenance of constant temperature, illumination, and nutrient supply throughout the investigation. The temperatures reported are those of water pumped through the glass jacket surrounding the culture chamber. The light intensity was measured at the surface of the culture unit with a Weston illumination meter. The light source consisted of four banks of incandescent lamps placed on four sides at a distance of

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nine inches from the culture unit. A continuous illumination was employed to obtain steady-state algal suspension. Synchronized cells of a known age were obtained with the technique described by Sorokin (1957) and Sorokin and Myers (1957). Density of the algal suspension was kept at one cubic millimeter of packed volume of cells per milliliter of culture medium. In grams per liter the composition of the culture medium was: KNO_3 —1.25, KH_2PO_4 —1.25, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ —1.00, CaCl_2 —0.0835, H_3BO_3 —0.1142, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ —0.0498, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ —0.0882, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ —0.0144, MoO_3 —0.0071, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ —0.0157, $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ —0.0049, ethylenediamine-tetraacetic acid (a chelating agent)—0.5. The pH of the medium was 6.8.

To prepare an algal suspension for photosynthetic measurements, cells were harvested from the culture chamber, centrifuged, and resuspended in a certain volume of the three salt medium containing the same amounts of KNO_3 , KH_2PO_4 and MgSO_4 as the culture medium. The pH of the suspending fluid was adjusted to 4.5 with KOH. The cells were kept suspended by bubbling with a 5.5 per cent CO_2 -in air mixture. Four milliliters of this suspension were measured into rectangular manometric vessels of about 9 milliliters capacity. Vessels were then gased with the 5.5 per cent CO_2 -in air mixture. Because of the large differences in photosynthetic capacity of cells grown under different conditions, the density of algal suspensions prepared for photosynthetic measurements and therefore the amount of cells per vessel was of necessity different in different experiments.

Illumination of the vessels was provided by a bank of incandescent lamps, with a total capacity of 1,340 watts, placed beneath the glass bottom of the water bath. This arrangement provided an illuminance of more than 2,000 foot candles at the level of the reaction vessels and proved to be sufficient for light saturation of photosynthesis. Different levels of illuminance were achieved by attaching neutral filters of known transmitting capacity to the bottoms of the reaction vessels.

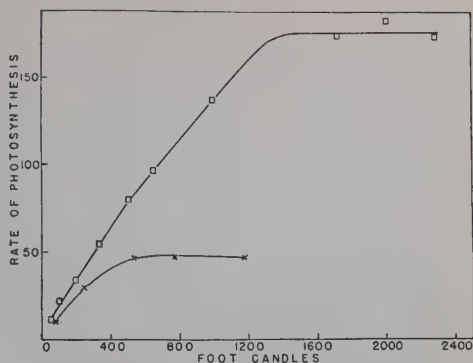
Measurements were made with the indirect, one-vessel method and a separately obtained assimilatory quotient (determined with the indirect two-vessel method) was used in calculations of the photosynthetic rates. The photosynthetic rates in this paper indicate oxygen evolution in units of volume of gas produced per unit of packed volume of algal cells per hour. No correction for respiration was made; the data, therefore, represent apparent photosynthesis.

Results and Discussion

The dependence of photosynthetic activity on light intensity during photosynthetic measurements, with temperature as a parameter, is shown in Figure 1. When measured at the higher temperature, the photosynthetic rates, both in light-independent and light-limited portions of the light intensity curve, were considerably higher than those determined at the same temperature maintained during culture in the growth chamber. At 39°C the light saturation rate was almost 4 times higher, and the light saturating intensity approximately 3 times higher than the corresponding values at 25°C .

The conditions of growing cells for photosynthetic measurements are subject to argument. Curves 1 and 2 in figure 2 illustrate the effect of light intensity during the previous period of culture in the growth chamber. At

Figure 1. Rates of apparent photosynthesis in $\text{mm}^3\text{O}_2/\text{mm}^3$ packed cells/hour as dependent on light intensity during photosynthesis measurements of *Chlorella* 7-11-05. Cells grown under 60 foot candles and at 25°C . The upper curve (squares) gives the rates measured at 39°C ; the lower curve (crosses) indicates the rates measured at 25°C .



39°C , the light saturation rate for photosynthesis is more than 3 times higher for cells cultured at 400 than those at 60 foot candles. The light-saturating intensity also seems to be lower for the cells grown at 60 foot candles. However, the transition between the light-limited and the light-independent portions of the light intensity curve for cells grown at 60 foot candles is so gradual that a determination of the saturating intensity is difficult. The difference in photosynthetic capacity between cells grown at different light intensities resembles that for sun and shade plants.

It is well known that under natural conditions sun plants differ from shade plants by a higher photosynthetic capacity at light intensities above the light saturation point and by a lower photosynthetic rate at lower light intensities (Lubimenko, 1905; Boysen-Jensen, 1918; Lundegårdh, 1921). It has even been shown that the same difference can be observed between parts of the same plant kept either in sun or in shade (Boysen-Jensen and Müller, 1929). Sargent (1940) and Myers (1946 b) were also able to show that the photosynthetic characteristics of *Chlorella pyrenoidosa* depend on the intensity of illumination during the previous period of culture. Sargent (1940)

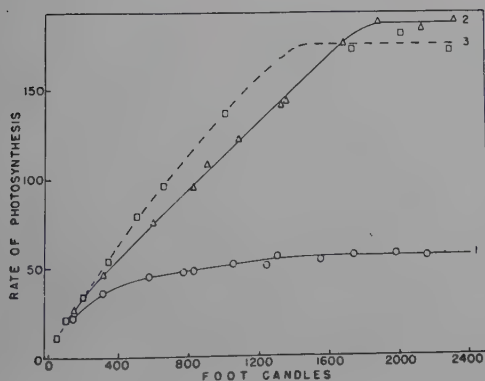


Figure 2. Rates of apparent photosynthesis in $\text{mm}^3\text{O}_2/\text{mm}^3$ packed cells/hour as dependent on light intensity during photosynthesis measurements of *Chlorella* 7-11-05. Curve 1 (circles) gives the rates for cells grown under 60 foot candles and 39°C ; curve 2 (triangles) describes the rates of cells grown under 400 foot candles and at 39°C ; curve 3 (squares) shows the rates of cells grown under 60 foot candles and at 25°C . All measured at 39°C .

grew cells at two light intensities and found that the maximum photosynthetic rate was higher for cells grown at the higher light intensity. Myers' observations extended over a much larger range of light intensities. He reported that with an increase in light intensity (to 35 foot candles) the photosynthetic capacity of cells increased, as measured at light and CO_2 saturation; then with a further increase in light intensity the photosynthetic capacity began to fall. However, with *Chlorella* cells, neither Sargent nor Myers were able to demonstrate a higher photosynthetic rate in the light-limited region of the light intensity curve for cells grown under lower light intensity. In Sargent's experiments photosynthetic activity of cells grown under weaker illumination was also at lower light intensities lower than of those grown under stronger illumination. Myers pointed out the inconsistency of the phenomenon. In certain of his experiments cells grown at lower light intensities had higher photosynthetic rates, while in other experiments cells grown at lower light intensities had lower rates.

A key to the discrepancy in the photosynthetic characteristics of sun and shade plants grown under natural conditions and those of algal cells cultured under controlled conditions at different light intensities, is supplied by a consideration of the importance of temperature in such studies. Curves 1 and 3 in figure 2 show that for algae experienced the same light intensity but different temperatures in the growth chamber, both light-limited and light-saturated rates of photosynthesis were considerably higher in cells grown at 25°C and measured at 39°C than in cells grown and measured at 39°C .

A comparison of two pairs of curves, 2 and 3, with 1 and 2, in figure 2 reproduces differences in experimental set up in studies of sun and shade plants in natural habitats on one hand and of algal suspensions grown at different light intensities under controlled conditions on the other. The relationship between curves 1 and 2, which is similar to that observed by Sargent (1940) and Myers (1946 b), indicates the influence of different light intensities during the growth period on the photosynthetic capacity. The situation presented by curves 2 and 3 reproduces conditions encountered in studies of sun and shade plants. In their natural habitats shade plants are generally subjected also to lower temperatures than the sun plants. The maximum photosynthetic activity above light saturation point is slightly higher in cells cultured at higher illumination and higher temperature (curve 2). However, the slope of the light-limited portion of the curve is substantially steeper in cells cultured at lower light intensity and lower temperature (curve 3). As a result of the higher efficiency of utilization of incident energy by cells grown at lower illumination and lower temperature these cells have a photosynthetic rate in the light-limited region up to 20 per cent higher than cells grown at the higher light intensity and the higher temperature.

An attempt could be made to account for the increase in the photosynthetic capacity of cells, under the influence of certain temperature and light conditions during the growth period, by a higher concentration of pigments and hence a higher absorption of incident light. However, this explanation does not find much experimental support. It has been shown by Warburg (1928), Sargent (1940), Myers (1946 b), and Oorschot (1955) that cells grown at the same temperature but at a higher light intensity have a lower chlorophyll content. Therefore, the generally observed higher photosynthetic capacity for cells grown at higher light intensities cannot be explained by a higher absorption of the incident light energy.

Studies on the influence of temperature showed that a higher temperature generally favors chlorophyll formation though the magnitude of the effect differs with different temperature ranges. Lubimenko and Hubbenet (1932) found that between 5° and 15°C there was a ten-fold increase in the rate of formation of chlorophyll, while between 18° and 28°C the increase was only by a factor of 1.5—2. Oorschot (1955) observed an increase in chlorophyll concentration of 50 per cent between 20° and 30°C, but practically no difference between 30° and 40°C. Thus, a decrease in the maximum photosynthetic rate in cells grown at higher temperature is generally accompanied by a higher formation of chlorophyll and, therefore, cannot be explained as the result of a lower absorption of the incident light energy.

A lower photosynthetic capacity of cells grown at a higher temperature could be the result of inhibitory agents produced by the cells if the formation of these agents was favored by a higher temperature. Pratt (1940) demonstrated the production of a growth-inhibiting substance by *Chlorella vulgaris*, and showed the inhibiting effect of *chlorellin* on photosynthesis (Pratt, 1943). The only attempt to study the dependence of the accumulation of *chlorellin* in culture medium on the external conditions did not produce convincing results (Spoehr *et al.*, 1949). In other experiments, by the same group, on the effect of temperature on the concentration of *chlorellin* in dried, ground *Chlorella* cells, the favorable effect of a higher temperature was clearly indicated during photooxidation. The possibility of a similar process under certain conditions also in living cells cannot be excluded.

More convincing evidence has been obtained on the way in which light intensity during the period prior to photosynthetic measurements can affect the developmental status of an algal suspension and through this the photosynthetic capacity of algal cells. It has been shown (Tamiya *et al.*, 1953, Nihei *et al.*, 1954; Sorokin and Myers, 1954) that in the course of their development algal cells undergo cyclic changes in their photosynthetic activity. Sorokin (1957) obtained light intensity curves of photosynthetic activity of cells of different developmental stages. The light intensity curves for 0,

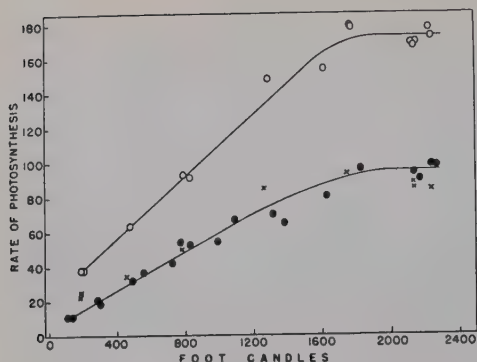


Figure 3. Rates of apparent photosynthesis in $\text{mm}^3\text{O}_2/\text{mm}^3$ packed cells/hour as dependent on light intensity during photosynthesis measurements of *Chlorella* 7-11-05. Cells before being taken for photosynthesis measurements were synchronized by light: dark regimen (Sorokin, 1957). Filled circles indicate zero time cells (from dark), open circles — cells three hours old; crosses — cells 9 hours old. Curves for the zero and three hour cells taken from Sorokin (1957).

3, and 9-hour old cells — all grown and measured at the same temperature of 39°C — are depicted in figure 3. The relationship between these curves is reminiscent of those between light intensity curves of cells from steady-state suspensions grown at different light intensities. With the progress in cell development both the photosynthetic activity of cells in the light-dependent portion of the light intensity curve and the maximum photosynthetic capacity in the light-saturated region of the curve first increase (3 hour cells over 0 hour cells) then fall (9 hour cells compared with 3 hour cells). That this reminiscence might be not incidental is suggested by the observations on the effect of the intensity of illumination on the average developmental status (age) of steady-state algal suspensions. It has been reported that, on an average, cells grown at higher light intensities are larger. (Myers, 1946 a; Tamiya *et al.*, 1953). Observations on the life cycle of strain 7-11-05 indicated that at higher light intensities cells grew larger and produced more autospores from one mother cell than under lower light intensities. It should be expected that steady-state algal suspensions grown at higher light intensities would consist of a larger proportion of cells in more advanced developmental stages than the suspensions grown at lower light intensities. Changes in photosynthetic characteristics of cells grown at different light intensities would be then at least partially due to the shifts in average developmental status of cells with the increasing light intensity. Some other cellular characteristics in steady-state suspensions change regularly with the increase in light intensity: cells become larger, the dry weight of a packed volume of cells (probably also their specific gravity) increases, and the chlorophyll content per dry weight of cells decreases (Warburg, 1928; Sargent, 1940; Myers, 1946 a). It is interesting that in synchronized algal suspensions these cellular characteristics also change in the same direction during cell development (Table 1).

There have been frequent attempts to correlate algal growth with photosynthetic characteristics or to determine the efficiency of utilization of light

Table 1. *Cellular characteristics in the course of cell development in the high-temperature strain, Chlorella 7-11-05.*

Developmental stage of cells in hours	Volume of a cell in cubic microns	Dry weight, mg/mm ³ , of packed cells	Chlorophyll content in relative units/mg dry weight
0	17	0.207	0.151
3	42	0.278	0.109
6	128	0.297	0.108
9	384	0.317	0.095

energy in over-all growth process on the basis of manometrically obtained photosynthetic measurements. It has been emphasized that the maximum photosynthetic capacity usually, if not universally, exceeds the growth capacity and that cells possess a mechanism of disposing of the excess of carbon fixed in photosynthesis but not used in the growth process. In this sense any photosynthesis-saturating light intensity was called "unphysiological" (Myers, 1946 b). It is clear, however, that maximum photosynthetic capacity determined under light-saturating conditions in manometric experiments has no constant relationship to the growth rate of the culture during the period prior to photosynthetic measurements. This is illustrated by data in Table 2 where the growth rate of the culture and the corresponding maximum photosynthetic capacity of cells, as determined in manometric experiments, are given for a number of different sets of growth conditions. A photosynthesis-saturating light intensity may or may not be "unphysiological" depending on the conditions under which the cells were grown.

From the results of photosynthetic measurements, Myers (1946 b) attempted to calculate the photosynthetic rates actually prevailing in the cultures used for manometric measurements and to determine from these data the light-saturating intensity. Owing to the differences in the source and geometry of illumination, however, it is impossible to predict which measure of light intensity during manometric experiments corresponds to the light intensity received by an individual cell in a culture. Differences in the mutual shading by cells, due to the differences in the density, the thickness of the

Table 2. *Light saturation rates of photosynthesis for cells of Chlorella 7-11-05, grown under different conditions.*

Conditions of growing cells			Rate of photosynthesis, mm ³ O ₂ /mm ³ cells/hour, measured at	
Temp. °C	Light intensity, foot candles	Growth rate, number of doublings per day	25 °C	39 °C
25	60	2.3	47	175
39	60	2.0		56
39	400	9.9	50	186

layer of the algal suspensions, and to the degree of agitation, have not been successfully reduced to a simple equation. In attempting to account for the discrepancy, Myers (1953) revised his calculations of growth-saturating light intensity from 100 to 400 foot candles.

Differences in the amount of light energy received by an individual cell in the growth chamber determine not only the growth characteristics in the growth chamber but also the subsequent capacity of the cell to photosynthesize. This capacity seems to depend, at least partially, on the developmental stage of the cell. After transferring a steady-state algal suspension to different external conditions the growth and photosynthetic characteristics will change, the adjustment being influenced by the induced changes in the developmental status of the algal suspension.

Summary

Cells of the high-temperature strain, *Chlorella* 7-11-05, were grown under different conditions of temperature and light intensity. Their photosynthetic activity, as affected by the past history of the cells and environmental conditions (temperature and light intensity) during photosynthetic measurements, was studied with the Warburg technique. It was observed that light intensity curves both in light-limited and light-independent regions of the curves largely depended on the conditions for growing the cells. The photosynthetic characteristics of cells grown at lower light intensity and lower temperature were related to those known for shade plants; the photosynthetic characteristics of cells grown at higher light intensity and higher temperature were found to be comparable to those known for sun plants. There was no direct relationship between the growth rate of an algal culture in the growth chamber and its photosynthetic performance in the Warburg vessels. The possibility of the differences in photosynthetic characteristics of cells with different past history being due to the differences in developmental status of algal suspensions, as affected by environmental conditions, was discussed.

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Auxins and Growth-Inhibiting Substances in Maize Kernels

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Introduction

Several investigators have studied auxin conversions in cereal grains during the period from the formation of the kernel until its maturation, as well as during the swelling and germination of the ripened kernels.

The fact that the content of extractable auxin in the cereal grains is greatest prior to maturation and falls as this process advances has been demonstrated by, among others, Hatcher and Gregory (1941) and Avery, Berger and Shalucha (1942 b). Hatcher (1943) found that in connection with the disappearance of the auxin an inactive auxin precursor is formed. Avery, Berger and Shalucha (1942 a) showed that the major part of the auxin in the maize kernel does not occur in the free state but is present as a precursor. They also demonstrated, however, that the amount of precursor is greater in unripe maize than in ripe resting maize.

These investigations of the auxin balance in maize and other cereal grains have been carried out with aqueous extracts of the kernels. Extraction with water yields considerably more auxin than extraction with ether, due to the fact that only certain auxin fractions or auxin precursors are extractable with ether. The auxin occurs partly free and partly bound, probably with proteins (Siegel and Galston, 1953, and Galston, 1956). Ether extraction for very short periods, a few hours, yields chiefly the free auxin. On continued extraction for a longer time, and preferably at temperatures around 30°C, auxin is obtained, the bound auxin, that has been liberated during the course of the extraction from its linkage or formed from a precursor (van Overbeek *et al.*, 1947, and Hemberg, 1951, 1954 a and b, 1955).

IAA has been isolated from maize by Berger and Avery (1944). (The reader is referred to the end of this chapter for the abbreviations employed here). They also found another indole derivative in maize, which contains the C=O group, probably in a COOH group. IAEt has been isolated from unripe maize by Redemann *et al.* (1951). Kefford (1955) isolated from the milk stage of maize kernels several substances active in the straight growth test, partly IAA, partly accelerator α . In chromatograms run in isopropanol — ammonia — water, accelerator α is found between the spot of application of the solution and the IAA spot. Kefford also found a substance with auxin action, which in the same chromatographing solvent moves farther than IAA and is located closer to the front of the solvent. He believed that this substance was IAN and not IAEt. He has demonstrated the substance in the acid fraction of the extract where the neutral IAN can also be found but hardly IAEt.

Stowe and Thimann (1954) have with acetone isolated from maize several indole compounds active in the straight growth test. In chromatographing the acid fraction of the extract in isopropanol — ammonia — water, they find: one substance, X_4 , with a Rf value of 0.12, which they assume to be IPyA; a second substance with a Rf value of 0.25, which is IAA; a third substance, X_3 , with a Rf value of 0.52. All these substances are acid. In addition they find a neutral substance, X_2 , with a high Rf value. This substance does not seem to them to be identical with IAN, nor is it IAEt. According to Bentley, Housley, and Britton (1956), the X_4 found by Stowe and Thimann cannot be identical with IPyA, since this substance is decomposed by chromatographing in isopropanol — ammonia — water. Cartwright, Sykes and Wain (1956) have found mainly IAA in chromatograms of ether extracts of maize kernels. From the figures in their work the presence of exceedingly small amounts of other substances can be inferred, but they themselves do not mention this in their paper. The X_3 found by Stowe and Thimann, however, has been earlier discovered by Lexander (1953) in extracts of wheat roots. In the area for X_3 in the chromatograms she was sometimes able to find an indication of growth stimulation, sometimes not. Yamaki and Nakamura (1952) showed that IAAlD is present in maize kernels.

The changes in the auxin content of extracts of maize kernels during different stages of swelling have been studied by, among others, Avery, Creighton and Shalucha (1940), von Guttenberg and Lehle-Joerges (1947) and Hemberg (1955). Cartwright, Sykes and Wain (1956) have investigated by means of paper chromatography the changes in the content of IAA in ether extracts of maize in connection with the swelling. On that occasion no division into free and bound IAA has been made, nor have they studied the occurrence of eventual neutral auxins.

Hemberg (1955) showed that if maize is allowed to swell in water containing IAA, this is absorbed and is found in the kernels, partly as free auxin, partly as bound. According to Siegel and Galston (1953) and Galston (1956) a part of the absorbed IAA is linked with protein, while a part is decomposed by oxidation. In pea seedlings, according to Andreae and Good (1955) and Andreae and Ysselstein (1956), exogenous IAA combines with aspartic acid to form indoleacetylaspatic acid. Good *et al.* (1956) have found that also other substances can be formed by plants from exogenous IAA. Whereas indoleacetylaspatic acid is formed above all in pea and onion, IAAM is formed in grass coleoptiles.

Growth-inhibiting substances occur very often in plant tissues. For further literature on these substances the reader is referred to Hemberg (in press). One of the most commonly occurring inhibitory substances is the acid compound inhibitor β . From a figure in Kefford's (1955) work it can be inferred that this inhibitor probably occurs also in maize kernels

In the present paper an account will be given of the occurrence of free and bound auxin in maize kernels at different stages of maturity. Furthermore it will be stated which acid and neutral auxins or auxin precursors as well as which inhibitors are found in the free and in the bound auxin fractions and also how the addition of IAA to maize kernels affects the content of IAA and its precursors.

The following abbreviations will be employed: indoleacetic acid=IAA, indolepyruvic acid=IPyA, indole acetaldehyde=IAAld, indole ethylacetate=IAEt, indole ethyl alcohol=IEt, indole acetamide=IAAM, indole acetonitrile=IAN.

Methods

Test methods. The Avena curvature test has been used in the analysis of the total auxin content in maize kernels, and the same technique has been followed as in earlier investigations (Hemberg, 1947 and 1955). In the biological analysis of paper chromatograms the straight growth test has been employed, with 5 mm long coleoptile sections of Avena cut 3 mm below the coleoptile tip. The coleoptiles have been cultivated in water-saturated vermiculite at 95 % relative humidity and 25°C. In the first experiments Victory oats were used, in the later ones the huskless variety Brighton.

For biological analysis the paper chromatograms were cut into 1 cm wide strips, which were placed in separate beakers. To these were added 4.0 ml citrate buffer (0.248 g. monopotassium citrate per liter). In the first experiments 16 g. glucose were added per liter, in later ones the glucose was omitted. On the other hand, in the later experiments 1 g. Tween 80 per liter buffer was added to facilitate the solution of the active substances (Nitsch and Nitsch, 1955, and Nitsch 1956). Ten coleoptile sections were placed in each beaker, and their growth was measured after 16—20 hours and compared with that of the control sections. The latter had lain

for the same length of time in buffer, to which had been added chromatography paper that had been in contact with the chromatographing solvent, but without the addition of the plant extract. The beakers were shaken during the entire experimental period in the first experiments, but in the later ones they remained still in the incubator.

In certain cases, where the extracts have been very rich in IAA or other indole compounds, it has been possible to develop the chromatograms by spraying with 2,4-dimethyl-paraaminobenzaldehyde (Ehrlich's reagent), *cf.* Nitsch and Nitsch (1955) and Nitsch (1956). Then, after they had been dried with a hot air fan and allowed to hang in the air for 30—60 minutes, the intensity of the different spots was measured by means of a photometer, model "EEL SCANNER". This had been calibrated with the help of chromatograms with known amounts of IAA and IAET. The method has great experimental errors; but since it in contrast to the biological method of determination is exceedingly rapid, it has been employed in certain experiments.

Chromatography. Ascending chromatography has been used. The chromatograms have been run in tubes, one chromatogram in each, according to Nitsch (1956). Each chromatographing paper was 2.5 cm wide and was allowed to hang in the chromatographing solvent until the solvent front had risen 15 cm above the spot of application. Primarily the following have served as chromatographing solvents: 70 % ethanol; isopropanol-ammonia-(sp. gr. 0.91)-water (100 : 14 : 6) or *n*-hexane in water-saturated air.

Great care has been exercised to free the chromatograms from every trace of chromatographing solvent before the biological assay. The chromatograms have been carefully dried with a hot air fan and then placed in a desiccator, which has been evacuated for at least half an hour by water suction.

Extraction, purification and fractionation of the extracts. Ether extraction has been employed for the separation of free and bound auxin. With free auxin is meant as in earlier works (Hemberg, 1951, 1954 a and b, 1955) the auxin which can be extracted with ether during the first three hours at +2—+4°C. With bound auxin is meant the auxin which in the continued extraction at +28°C for 45 hours is liberated from the kernels.

The extracts have been fractionated into acid and neutral fractions according to Larsen's (1955) modification of Boysen Jensen's (1941) method II. The neutral fraction contains considerable fat, which makes it very difficult to work with. In the application of the neutral extract to chromatographing paper, the fat affects the course of the chromatographing. In chromatographing smaller amounts of the neutral extracts, the difficulties can be partly eliminated by employing double chromatography. In this process the extract is chromatographed in 70 % ethanol; after drying and cutting away the fatty spot of application, the chromatogram is eluted with ether and transferred to a new chromatographing paper. With this technique, however, substances may easily be lost, partly in the operations themselves, partly in that substances with a *R_f* value of 0 are not included in the second chromatogram.

In order to free extracts from fat Nitsch and Nitsch (1955) have worked out a method of dissolving the neutral fraction in acetonitrile and shaking with *n*-hexane, which dissolves the fat but not the indole compounds. The method has been used in only a few experiments in the present investigation.

For examination of mainly the neutral auxins, however, another method of extraction has been worked out, which yields extracts without noticeable fat contents. In this method 100 maize kernels were ground in a mill (dry kernels) or crushed in a

Table 1. *Fractionation of 0.5 ml of an alcoholic solution containing 0.096 mg IAA and 0.098 mg IAET, partly by shaking with ether according to Larsen (1955), partly chromatographically with the aid of a kieselguhr column according to Schill and Ågren (1951). After fractionation the extracts were dissolved in 10 ml ether. The fractionated extracts have been chromatographed on paper and the chromatograms sprayed with Ehrlich's reagent. The intensity of the coloured spots has been measured in a photometer, and the results have been compared with calibration curves for synthetic IAA and IAET.*

Extract no.	Method of fractionation	Fraction	Solution per chromatogram ml	IAA		IAET	
				Found μg	Calc. μg	Found μg	Calc. μg
1001 b ₁	Shaking with ether	Neutral	1.0	0	0	10	9.8
			2.0	0	0	20	19.6
1001 b ₂	" " "	Acid	1.0	11	9.6	0	0
			2.0	23	19.2	0	0
1002 b ₁ 1	Kieselguhr column	Neutral	1.0	0	0	6	
1002 b ₁ 2	" "	"	1.0	0	0	5	
1002 b ₁ 3	" "	"	2.0	0	0	0	
1002 b ₁ 4	" "	"	2.0	0	0	0	
						11	9.8
1002 b ₁ 1 b ₁ 2 b ₁ 3 b ₁ 4	" "	"	1.0 of each	0	0	12	9.8
1002 b ₂ 1	" "	Acid	1.0	9		0	
1002 b ₂ 2	" "	"	1.0	0		0	
1002 b ₂ 3	" "	"	2.0	0		0	
1002 b ₂ 4	" "	"	2.0	0		0	
				9	9.6		

mortar (swelled kernels) and transferred to a 100 ml Erlenmeyer flask. To the ground kernels were added 50.0 ml phosphate buffer, pH 7.2. The flask was shaken for one hour, and thereafter the contents were filtered through cloth and centrifuged. Then 5 ml of the supernatant liquid were pipetted into each of a number of beakers. Thereafter 2—3 drops of methylorange solution were added to each beaker as well as 3.5 M phosphoric acid, so that the pH became ca 2.7. To each beaker 14.0 g. kieselguhr, Hyflo super cel, were added and mixed well with the liquid. The mixture from each beaker was packed in a glass column (*cf.* Schill and Ågren, 1951), through which 200 ml ether saturated with phosphate buffer, pH 2.7, were allowed to pass. The ether, which after the passage through the column contained the neutral and acid ether-soluble substances in the extract, was evaporated and fractionated in the usual manner into neutral and acid fractions. Since maize kernels of varying degrees of swelling contain different amounts of water, this will, of course, affect the quantitative yield to a certain extent. Despite this discrepancy the extraction method can be used to give an idea of the approximate amounts of the different auxins in the kernels and of which auxins are present.

In order to test whether the extraction method destroyed the auxin in any way, 0.5 ml of an alcoholic solution containing 0.096 mg IAA and 0.098 mg IAET was added to 5 ml phosphate buffer, pH 8.8, and mixed with 10 g. Hyflo super cel. After a column had been packed with this mixture, 4 portions of 50 ml. ether saturated with the same phosphate buffer were allowed to pass through the column. In this ether only IAET should be found but not IAA. Each portion was evaporated separately and

Table 2. *Amount of IAA in extracts of maize kernels (Weibull's Mette).* The extracts were prepared partly with ether, partly with phosphate buffer, pH 7.2, see the text. The extracts were fractionated and the acid fraction chromatographed. The chromatograms were sprayed with Erlich's reagent, and the IAA spots were measured in a photometer.

Method of extraction	IAA per kernel μg	Total IAA per kernel μg	IAA per kg maize mg
Ether extraction Free auxin	0.06 0.07 0.08	0.30	1.8
Ether extraction Bound auxin	0.26 0.23 0.20		
Phosphate buffer extraction	3.3 3.7 4.1	3.7	22.7

dissolved in 10 ml ether. Chromatograms were made with the different extracts and sprayed with Ehrlich's reagent. The colour intensity of the IAA spots thus obtained was determined with a photometer. All the IAA was found in the first two ether portions (*cf.* Table 1, extract no. 1002 b_1 — b_4). Thereafter the kieselguhr was removed from the glass column as quantitatively as possible and acidified with 3.5 *M* phosphoric acid until the pH became 2.7. A new glass column was packed with this mixture, and then 4 portions of 50 ml ether saturated with phosphate buffer, pH 2.7, were allowed to pass through. These were also evaporated separately and each dissolved in 10 ml ether. Thereafter chromatograms were made also with these extracts. The chromatograms were sprayed with Ehrlich's reagent and the intensity of the spots measured (*cf.* Table 1, extract no. 1002 b_2 — b_4). All the IAA was recovered in the first portion of ether. Simultaneously with this fractionation, a fractionation of the same amounts of IAA and IAA was carried out by shaking these substances dissolved in 25 ml ether with saturated glucose solution according to Larsen (1955). The amounts of recovered IAA and IAA were determined in the same way as in the foregoing experiment (*cf.* Table 1, extract no. 1001 b_1 and 1001 b_2). This fractionation was found to give about the same results as the one with glass columns, and therefore the latter method may be regarded as quantitatively satisfactory. The somewhat higher values for IAA obtained by shaking with ether lie entirely within the limits of error of the method of determination.

However, since in fractionation according to the column method it proved necessary to remove the contents of the column and after acidification make a new column, whereby losses may, of course, easily occur, in the further work with the extraction of maize with phosphate buffer the extracts were acidified before they were mixed with the kieselguhr in the manner previously described. Both the acid and the neutral auxin have therefore been found in the ether that has passed through the column. The column method has been employed to transfer the active substances into the ether without frothing.

If the yield of IAA from the extraction of maize with ether (free and bound auxin) is compared with that obtained with phosphate buffer and subsequent extraction in glass columns, it is found that the latter method gives considerably more IAA than the former (see Table 2). This is probably due to the fact that in the ether extrac-

tion only certain fractions of IAA are obtained, partly the free and partly the protein-bound; whereas in the extraction with phosphate buffer other precursors are also obtained, which are converted during the course of the extraction into IAA. Avery, Berger and Shalucha (1942 a) have also obtained more auxin by extraction with water than with ether. Therefore in the examination of the phosphate buffer extract, one must not overlook the possibility that the substances found there may have been formed during the course of the extraction and have not occurred in the plant tissue prior to the extraction.

Contents of Free and Bound Auxin in Maize Kernels of Varying Degrees of Development

In this part of the investigation maize of the variety Hardy Canada Gold was employed. Extractions were carried out on August 23, 1955 (milk stage), August 30 (early dough stage), September 6 and 22, October 11 and 25 and November 16. The maize was cultivated at W. Weibull's A.B. in Landskrona and was sent from there the day before each extraction. On all occasions the maize was received as unthreshed ears except on November 16, when it was threshed. At each extraction time two extracts were prepared, each from different ears. The lowest row of kernels on each ear was removed, and thereafter the 50 lowest remaining kernels were taken for an extract. On November 16 only one extract was prepared.

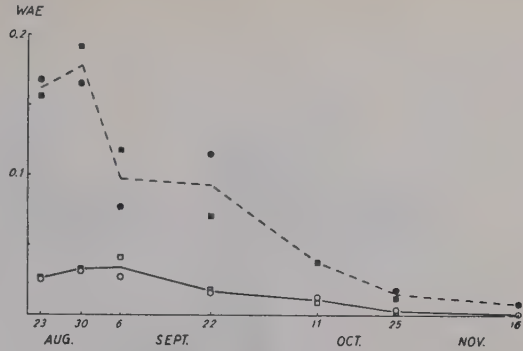
Prior to the extraction the kernels were crushed in a mortar or, if they were dry enough, ground in a mill. Although the extracts were fractionated into neutral and acid fractions and both fractions were examined, only the auxin content of the acid fraction will be accounted for in the following. The fat content of the neutral fraction had a disturbing effect on the analyses. It is true that by milk treatment of the neutral fraction some acid auxin could be formed, which indicates the presence of IAAd in the neutral fraction. The liberated amounts, however, were small, and the quantitative yield, due to the detailed method and the fat content of the extracts, was extremely variable.

The results, which are evident from Figure 1, show that the content of free as well as of bound acid auxin is higher during the earlier stages of development of the kernels than during the later ones. The content of bound acid auxin is very high during the milk and early dough stages, but thereafter it falls markedly. When the kernels are fully ripened, it is only $1/20$ of what it was during the milk stage. During the continued storage of the ripe kernels, according to Hemberg (1955) the content of the bound auxin further decreases.

Also the content of free acid auxin falls towards maturation. At full maturity it is only $1/30$ of what it was during the milk stage.

From Figure 1 it is evident that the content of bound auxin in the simultaneously prepared extracts varies very greatly. This is probably due to the

Figure 1. Amount of free (\circ and \square) and bound (\bullet and \blacksquare) acid auxin in ether extracts from maize kernels (variety Hardy Canada Gold) at different stages of maturity. \circ , \bullet and \square , \blacksquare represent different extracts prepared on the same time but from different ears. Abscissa: Date for extraction. Ordinate: Amount of auxin in WAE per kernel.



fact that the extracts are prepared from different ears. The degree of maturation of the ears may not have been the same, even if they were of uniform appearance. The ears may also have come from different plants.

The content of free acid auxin is always considerably lower than that of the bound. This indicates that a certain balance exists between the two auxin fractions with the point of equilibrium displaced towards the bound state. Since this gradually disappears during maturation, the reason is probably that the auxin is now converted into some precursor, from which acid auxin can no longer be liberated by prolonged ether extraction. This is more likely than that the auxin should be destroyed in connection with the maturation. As mature kernels swell, their content of bound and free auxin increases rapidly, indicating that auxin precursors are present in the kernels (Hemberg, 1955).

Paper Chromatographic Analysis of the Acid Fraction of Extracts from Maize Kernels of Different Degrees of Swelling

At first extracts containing acid growth substances from maize kernels (variety Hardy Canada Gold) swelled for 24 hours were subjected to chromatographic analysis. In order to obtain the extracts as pure as possible, they were chromatographed twice, first in 70 per cent ethanol and the second time in isopropanol—ammonia-water. From the histograms presented in Figure 2 it is evident that all extracts, those containing free auxin as well as those containing bound, exhibit a stimulative region between the R_f values 0.2—0.4. The stimulation is ascribed to IAA. Another stimulative region with higher R_f values, 0.5—0.8, is found in all histograms. The peaks in the histograms are, however, not as high for this substance as for IAA. In certain histograms a stimulation can be found within R_f 0.8—1.0 and in the histograms of free auxin a suggestion of a stimulation can be detected within R_f 0—0.05.

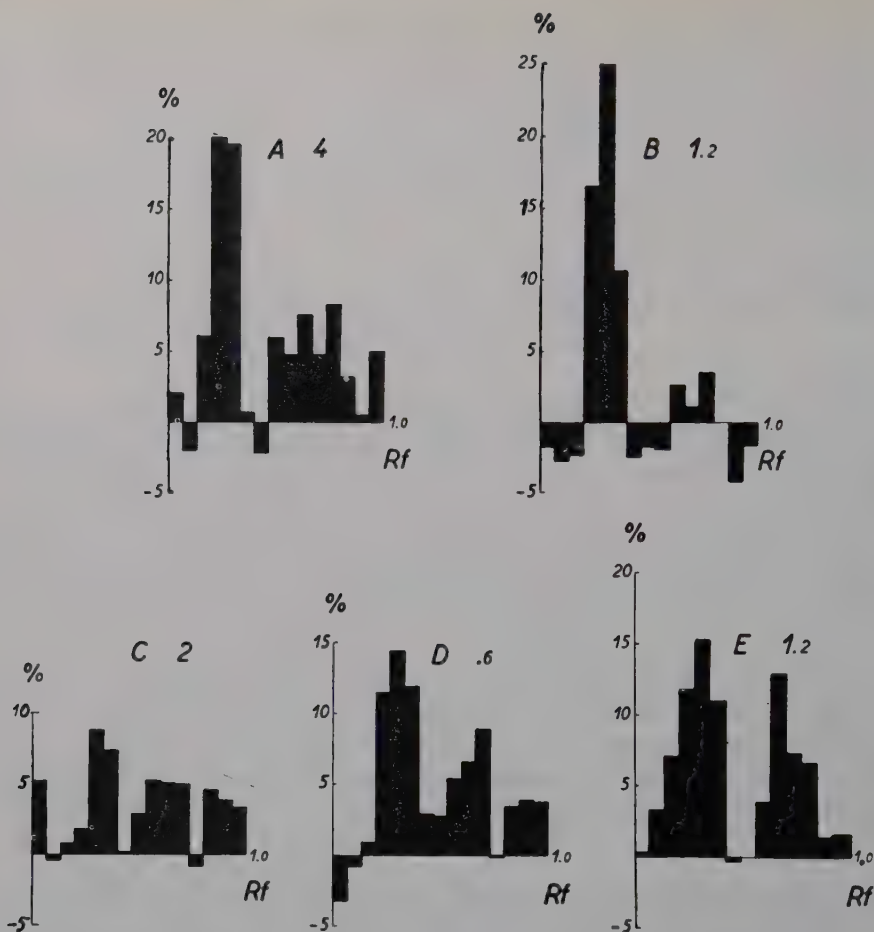


Figure 2. Biological assay of chromatograms of acid fraction of free (A and C), and bound (B, D, and E) auxin from ether extracts of maize kernels (Hardy Canada Gold) swelled for 24 hours. A and B represent one extract, C, D and E another extract. The chromatograms were at first run in 70 % ethanol and then in isopropanol—ammonia-water. The figures after the letters represent the number of maize kernels in the chromatograms. Abscissa: The position of the paper segment on the chromatogram in Rf units. Ordinate: Elongation of the *Avena* segments in per cent of the elongation of the control segments.

In Figure 3 are presented histograms from chromatographic experiments with extracts from dry maize kernels (variety Hardy Canada Gold) and from kernels swelled for 3 and 24 hours. In Figure 4 are shown histograms from similar experiments with the variety Mette, with dry kernels and kernels swelled for 3 hours. In this last experiment the extractions were made in duplicate each time and analyzed separately. In the histograms in Figures 3

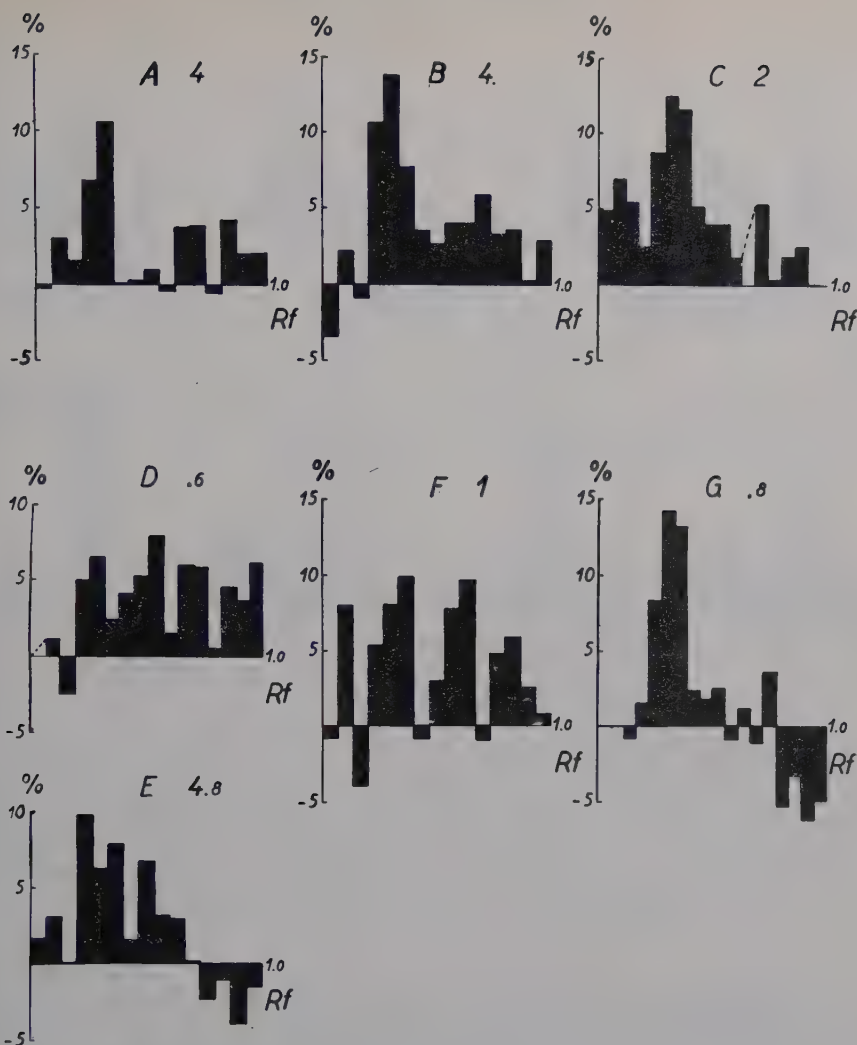


Figure 3. Biological assay of chromatograms of acid fraction of free (A, B and C) and bound (D, E, F and G) auxin of ether extracts from maize kernels (Hardy Canada Gold). A, D and E represent dry kernels, B and F kernels swelled for 3 hours, C and G kernels swelled for 24 hours. Further as in Figure 2.

and 4 the same stimulative regions are largely found as in figure 2: one region with low Rf value (*a*); one with the Rf value 0.2—0.4 (*b*=IAA); one with the Rf value 0.4—0.8 (*c*); and occasionally an additional one lying close to the solvent front (*d*). The histograms in Figure 4 include the position of IAA in simultaneously made chromatograms with synthetic IAA sprayed with Ehrlich's reagent.

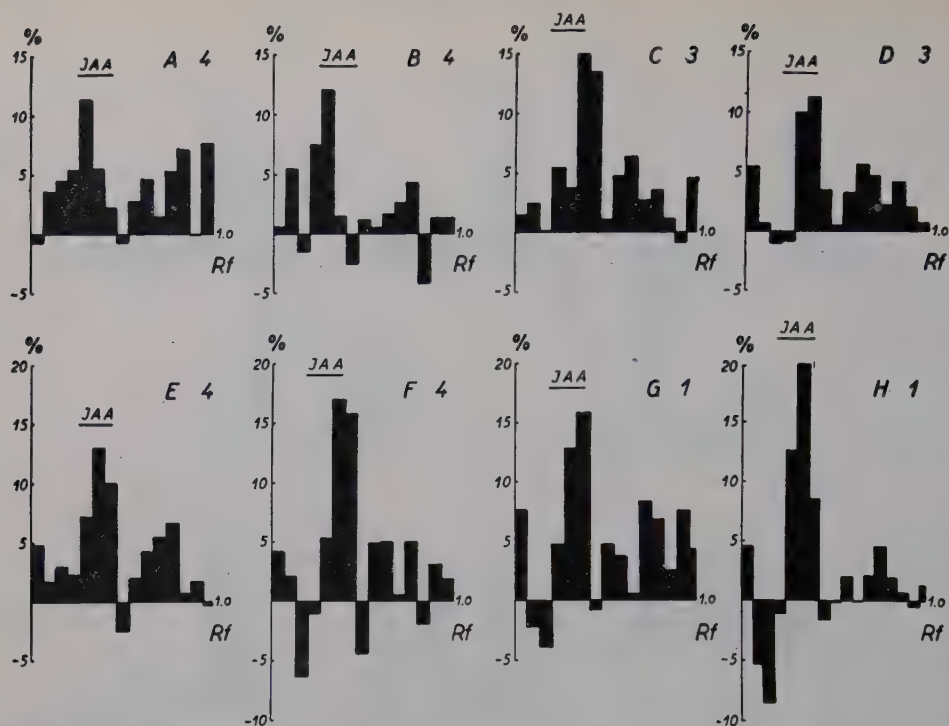


Figure 4. Biological assay of chromatograms of acid fraction of free (A, B, C and D) and bound (E, F, G and H) auxin from ether extracts of maize kernels (Mette). A, E and B, F represent extracts from dry kernels, C, G and D, H extracts from kernels swelled for 3 hours. The position of synthetic IAA in chromatograms run simultaneously and sprayed with Ehrlich's reagent is included in each histogram. Further as in figure 2 but the chromatograms were run only in isopropanol—ammonia-water.

The region *b* is most noticeable in Figure 3 as well as Figure 4. This may, of course, be due to the fact that the activity of the other substances in the biological test is considerably lower than the activity of IAA. It is, however, probably due above all to the fact that the other substances are present in much smaller amounts than IAA. Spraying the chromatograms of these extracts with Ehrlich's reagent gives evidence chiefly of IAA, even if a coloration in the position for *a* can be detected in the chromatograms. The region for the substance *c* is relatively strongly marked in the histograms. In Figure 3 D and Figure 4 A, F and G this region is divided into two areas. This division is not visible in all chromatograms, and therefore it does not necessarily imply that it is a question of two different substances. The division could have been caused by an especially strong concentration of inhibitory substance for some reason in the middle of the spot for *c*. The substance *a* gener-

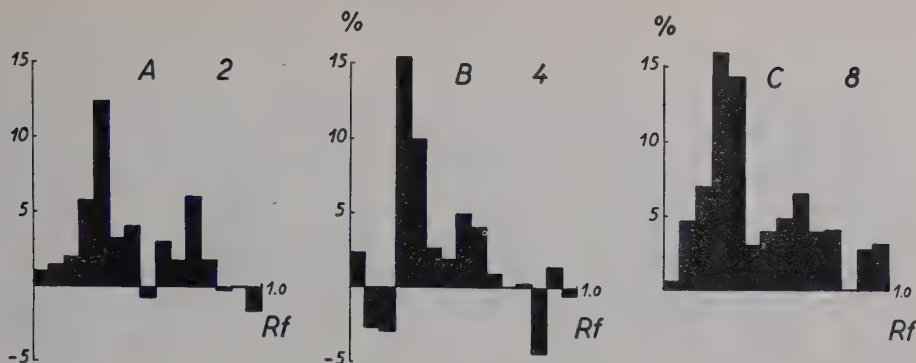


Figure 5. Biological assay of chromatograms of acid fraction of free auxin of ether extracts from maize kernels swelled for 24 hours (*Hardy Canada Gold*).

Further as in figure 2.

ally occurs in chromatograms of free auxin. In chromatograms of bound auxin *a* is found only in extracts from dry kernels or in extracts from kernels swelled for 3 hours, but not in extracts of kernels swelled for 24 hours. The substance has possibly been utilized during the swelling.

The substance *d* occurs occasionally in the chromatograms but is at times replaced by an inhibitory substance. Both substances probably occur in the extracts, and the dosage probably determines which one will dominate.

Figure 5 refers to chromatograms of different amounts of extract containing free auxin from kernels swelled for 24 hours. From the figure it is evident that the results of the test cannot be accorded too great quantitative significance. A twofold or fourfold increase of the extract amount affects the results very slightly.

From the foregoing experiments it is evident that one can demonstrate in ether extracts of maize kernels by means of biological tests the same substances that Stove and Thimann (1954) found with the help of chemical colour reactions in chromatograms from acetone extracts of maize. As far as can be judged from the chromatograms, it is only the amount of IAA that increases noticeably with the swelling of the kernels. All substances occur in the free as well as the bound fraction of the extracts. This implies that all the substances either can be adsorbed by other substances, for example protein compounds, from which they during the course of the ether extraction can be liberated or synthesized from precursors.

In Figure 6 some experiments are presented with the acid fraction of phosphate buffer extracts of maize of different degrees of swelling. From these experiments it is evident that the phosphate buffer extract contains mainly IAA, even if the presence of the other stimulative substances is infer-

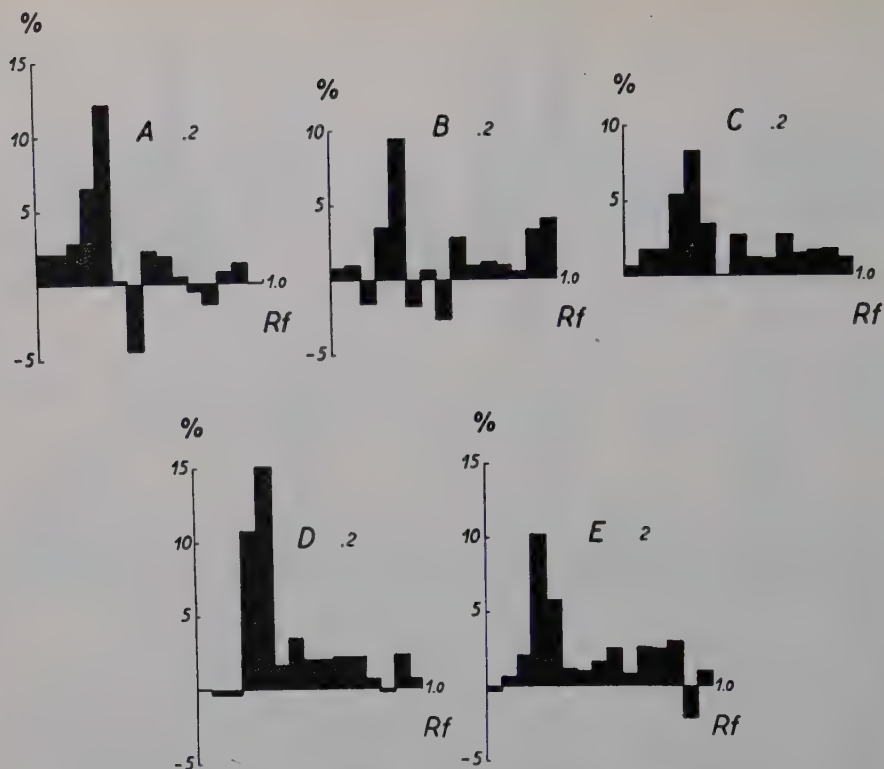
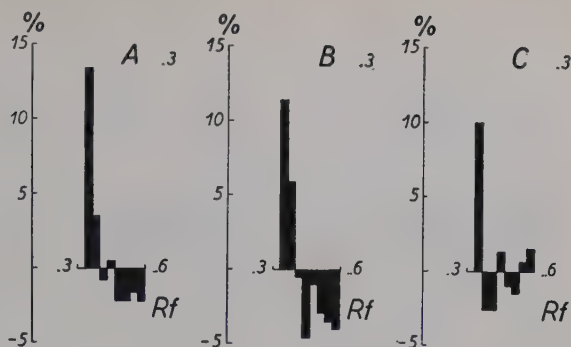


Figure 6. *Biological assay of chromatograms of acid fraction of phosphate buffer extracts from maize kernels (Mette). A and D extracts from dry kernels, B and E extracts from kernels swelled for 3 hours, C extract from kernels swelled for 24 hours. Further as in Figure 2 but the chromatograms were run only in isopropanol—ammonia-water.*

able from the chromatograms. The amount of IAA in these extracts is greatest in extracts of dry kernels and falls here during the course of the swelling. The result is thus directly opposite to that obtained with the ether extraction. The probable cause is that the phosphate buffer extraction draws out considerably more auxin, even precursors that are converted to acid IAA during the extraction. These precursors should be present in considerably greater amounts in dry maize than in swelled maize, where a large part has already been utilized in the formation of IAA, which has been consumed during the growth process. The results agree with those obtained by Avery, Creighton and Shalucha (1940). These investigators found in aqueous extracts of maize kernels more auxin in extracts of dry kernels than in extracts of swelled kernels. On the other hand, Guttenberg and Lehle-Joerges (1947) found less auxin in aqueous extracts of dry maize kernels than in aqueous extracts of

Figure 7. *Biological assay of chromatograms of acid fraction of phosphate buffer extracts from maize kernels (Mette) between the Rf units 0.33—0.60. A, dry kernels, B, kernels swelled for 3 hours, C, kernels swelled for 24 hours. Further as in figure 6.*



swelled kernels. In ether extracts of swelled maize kernels both groups of investigators found more auxin than in ether extracts of dry kernels.

Ether as well as water extracts of maize contain not only growth-stimulating substances but also growth-inhibiting ones. These could not, however, be found in all chromatograms. This is due to the fact that the stimulative substances in the chromatographing were able to spread out over such large parts of the paper that they overlap. A region with an inhibitory substance lying between two regions with stimulative substances in the chromatogram can thus be concealed in the biological test and can only cause a partial masking of the action of the stimulative substances. In the cases when inhibitory substances could be demonstrated, they were found at three different Rf values, namely: 0.1, 0.5, and 0.8—1.0. The inhibitory substance with the Rf value 0.5 is probably identical with inhibitor β (Bennet-Clark and Kefford, 1953). The substance occurs in ether extracts as well as phosphate buffer extracts of both dry and swelled kernels. In Figure 7 some chromatograms of phosphate buffer extracts of dry kernels and of kernels swelled for 3 and 24 hours have been analyzed between Rf 0.33—0.60 by cutting the chromatogram papers between these Rf values into 0.5 cm wide strips. Here the action of this inhibitory substance is clearly seen.

Paper Chromatographic Analysis of the Neutral Fraction of Extracts from Maize Kernels of Different Degrees of Swelling

Some chromatographic experiments with the neutral fraction from phosphate buffer extracts of dry maize kernels and of kernels that have been allowed to swell for 24 hours are presented in Figure 8. In order to obtain as pure extracts as possible, the extracts employed in the experiments accounted for in this figure have been further purified in the following manner. Extract of the neutral fraction corresponding to 8 kernels was evaporated to

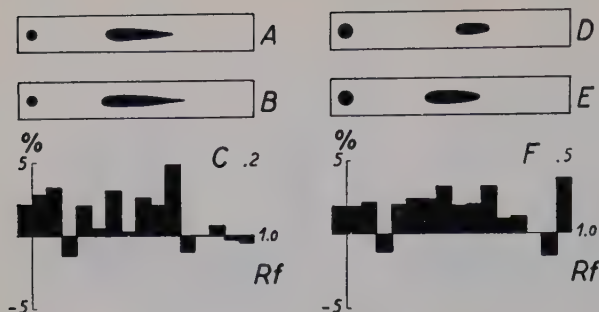
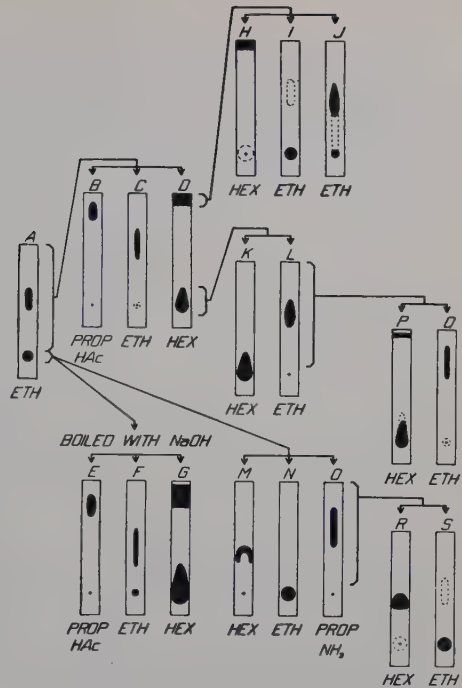


Figure 8. *Chromatograms of neutral fraction of phosphate buffer extracts from maize kernels (Mette). A, B and C dry kernels, D, E and F kernels swelled for 24 hours. A, B, D and E sprayed with Ehrlich's reagent. C and F analyzed biologically. The chromatograms run in 70 % ethanol.*

dryness in a 25 ml Erlenmeyer flask, and after the addition of 10 ml water the contents were shaken for 2 hours. Thereafter the contents were filtered through a glass filter (Jena 3G3), and the flask and filter were washed twice with 5 ml water each time. The combined water portions were alkalinized and shaken three times with ether. The ether will contain the neutral substances, now further purified from fat. The chromatograms were run in ethanol and sprayed with Ehrlich's reagent. In some cases they have been analyzed biologically. If we first regard the chromatograms sprayed with a chemical reagent, we find two different spots on these (see Figure 8, A, B, D and E). Both spots have the same blue-violet color which the reagent gives with IAA. One of the spots lies at the spot of application (*e*) and one has the *Rf* value 0.35—0.75 (*f*). The *Rf* value of the latter spot varies considerably in the different chromatograms, even at constant temperature and with exactly the same technique. The chromatographing paper, however, has not been buffered. The biological assays with chromatograms of these extracts indicate that *e* as well as *f* are active in the straight growth test (see Figure 8, C and F).

In Figure 9 some experiments with *e* and *f* are illustrated. These substances have been separated out of the neutral fraction by chromatographing in ethanol. One of the chromatograms has been sprayed with Ehrlich's reagent in order to show the position of the substances in the chromatograms (Figure 9 A). From the other chromatograms *e* and *f* have been eluted separately. The eluate from *e* has, among other things, been subjected to chromatography in *n*-hexane (Figure 9 M), in ethanol (Figure 9 N) and in isopropanol—ammonia-water (Figure 9 O). Each chromatogram has been sprayed with Ehrlich's reagent. The eluate containing *f* has been used for a number of chromatograms, of which some have been run in isopropanol—acetic acid-water (100 : 11 : 9) (Figure 9 B), some in ethanol (Figure 9 C) and others in *n*-hexane (Figure 9 D). One chromatogram of each has been sprayed with Ehrlich's reagent. In the ethanol chromatograms and to an even higher degree in the hexane chromatograms *f* has been divided into two different substances. In chromatographing

Figure 9. Chromatograms of neutral fraction of phosphate buffer extracts from maize kernels (Mette and Spangcross) run in different solvents. The chromatograms sprayed with Ehrlich's reagent. Black areas represent strong reaction, areas with dotted lines represent weak reaction, + denotes the spot of application. The different solvents are mentioned under each chromatogram: ETH=70 % ethanol; PROP HAC=isopropanol—acetic acid—water; PROP NH₃=isopropanol—ammonia—water; HEX=n-hexane in water saturated air.



in ethanol traces of a substance in the spot of application (*g*) are obtained, whereas the main part is found in the same place as earlier (*f*). In hexane *f* is obtained in the spot of application and immediately above this, while *g* is found close to the solvent front. On continued chromatographing in n-hexane of *f* obtained from the hexane chromatogram, only one spot appears, that for *f*. This is now located in and immediately above the spot of application (Figure 9 K). If the *f* obtained from the hexane chromatogram is chromatographed in ethanol, it is located in the same place as in earlier ethanol chromatographings (Figure 9 L, cf. A and C). The substance *g* on chromatographing in hexane still gives *g* quite close to the solvent front but also a small amount of *f* in the spot of application (Figure 9 H). On chromatographing in ethanol it has in one case remained in the spot of application and exhibited traces of *f* (Figure 9 I); in another case some *g* is found in the spot of application, but it is also largely converted into *f* (Figure 9 J). The substance *g* is thus very unstable and readily converted into *f*. It is probably formed from *f* in chromatographing in ethanol. This is demonstrated by the following experiment. The substance *f* obtained by hexane chromatography yields on renewed chromatographing in hexane only *f* (Figure 9 K). But if *f* obtained by hexane chromatography is subjected to chromatographing in

Table 3. Amount of the neutral substances *e* and *f* in phosphate buffer extracts of maize of different degrees of swelling and of different stages of maturity. The extracts were chromatographed and the chromatograms sprayed with Ehrlich's reagent. The amount of the different substances is expressed by stating the number of nonie units obtained in the planimetric measurement of the curves based on the chromatograms.

Maize variety	Hours of swelling	Swelling in water or IAA solution (100 mg per liter) for 3 hours	<i>e</i>		<i>f</i>	
			Number of kernels per chromatogram	Nonie units	Number of kernels per chromatogram	Nonie units
Weibull's Mette	3	H ₂ O	1.5	31	1.0 1.0	87 103
" "	3	IAA	1.5	58	1.0 1.0	94 79
" "	24	H ₂ O	1.5 2.0	58 75	1.0 1.0	65 61
" "	24	IAA	1.5 2.0	101 177	1.0 1.0	95 85
" "	48	H ₂ O	1.5	49	1.0 1.0	71 77
" "	48	IAA	1.5	55	1.0 1.0	105 93
Spancross	0	—	1.5	36	1.0 1.0	58 61
" "	3	H ₂ O	1.5	43		
" "	5	H ₂ O	1.5	80		
" "	24	H ₂ O	1.5	23		
" "	48	H ₂ O	1.5	16		
Spancross milk stage...	0	—	2.0	unmeasurable	1.0	unmeasurable
Spancross early dough stage	0	—	2.0 2.0	42 43	2.0 2.0	65 85

ethanol and the region containing *f* (Figure 9 L) is eluted and again chromatographed in hexane or ethanol, one obtains in addition to *f* also *g* (Figure 9 P and Q).

Also the substance *e*, which on chromatographing in hexane has an entirely different R_f value than *f* and *g*, is easily convertible into these substances. If *e* is chromatographed in the alkaline solution isopropanol—ammonia-water and the region containing *e* in the chromatograms (Figure 9 O) is eluted and chromatographed in *n*-hexane or ethanol, one finds in addition to *e* in these chromatograms also traces of *f* (Figure 9 R and S). If *e* is eluted from ethanol chromatograms and the eluate evaporated to dryness, boiled with 1-N NaOH for 30 minutes and thereafter fractionated, both *f* and *g* are found in the neutral fraction (Figure 9 F and G). If the neutral fraction of with NaOH

Table 4. *Rf* values in different solvents for the neutral indole compounds found in maize and for some known synthetic indole derivatives.

Substance	Rf values in			
	70 % ethanol	Isopropanol— ammonia—water	Isopropanol— acetic acid—water	n-Hexane 100 % humidity
<i>e</i>	0	0.40—0.80	—	0.30—0.45
<i>f</i>	0.35—0.75	—	0.80—0.90	0 —0.25
<i>g</i>	0	—	—	0.80—1.00
IAAld	0.45 (Yamaki & Nakamura, 1952)	—	—	—
IAEt	0.80 (Linskens, 1955)	0.70—0.90	—	0.70—1.00
IEt	0.70—0.90	—	—	—
IAAm	0.65—0.80	0.65—0.75	—	0
IAN	0.75—0.90	0.80—0.90	—	—
Tryptamine ...	0.63—0.85	0.75 (Linskens, 1955)	0.20—0.55	0 0.96—0.99 (Two spots)

boiled *e* is chromatographed in isopropanol—acetic acid-water, the same results are obtained as when *f* is chromatographed in this solution (Figure 9 E and B).

If maize is allowed to swell in water and is then extracted with phosphate buffer, the amount of *e* is seen to be temporarily increased, see Table 3. Whether the amount of *f* is also affected during the swelling cannot be stated at present, since the experimental errors in the determinations of *f* were very great. The amounts of *e* and *f* in Table 3 are stated by giving the nonie values that were obtained in planimetric measurement of the peaks of the curves based on the photometric examination of the chromatograms. From Table 3 it is also evident that the amount of *e* is less in maize in the milk stage than in the early dough stage.

A comparison of the *Rf* values of the substances *e*, *f* and *g* in different chromatographing solvents apparently shows no agreement with the corresponding *Rf* values of known synthesized neutral indole derivatives, which conceivably might occur in plants, see Table 4. Both *f* and *g* as well as *e* give a strong colouration with ninhydrin. Of the synthetic substances investigated, tryptamine gives a strong colour reaction with ninhydrin, whereas IAN shows a weak brown colouration and IAAM exhibits no colour whatsoever.

Since some preliminary chromatographic experiments with synthetic tryptamine — obtained by dissolving the hydrochloride in 1-N NaOH and shaking this solution with ether — indicated that the substance is divided into two on chromatographing in *n*-hexane, tryptamine has been subjected to further investigation. The results of this show that the *Rf* values of synthetic tryptamine on chromatographing in isopropanol—acetic acid-water and in

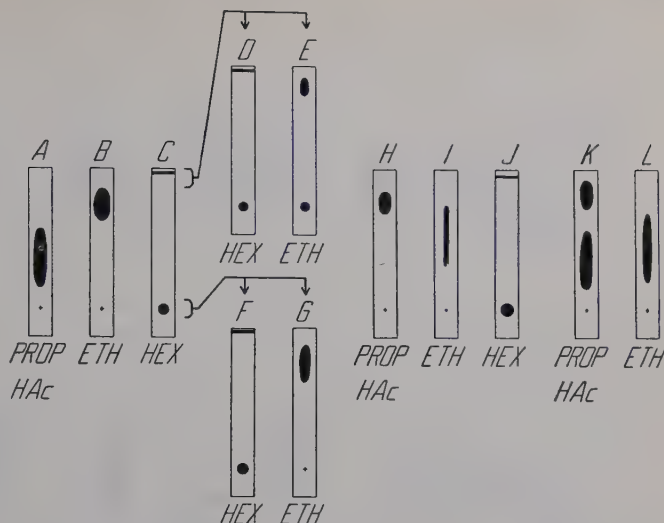
70 per cent ethanol differ from the corresponding Rf values of the substance *f*, (cf. Figure 10 A and B with Figure 9 B and C). On the other hand, tryptamine, as previously indicated, divides into two substances on chromatographing in *n*-hexane (see Figure 10 C). This is parallel to the conditions with the substance *f* (see Figure 9 D). But whereas *f* is divided into two substances on hexane chromatography only if the substance is first chromatographed in ethanol, this division of tryptamine takes place independently of whether the substance is first chromatographed in ethanol or not. The upper spot in hexane chromatograms of tryptamine gives on chromatographing in *n*-hexane as well as ethanol two spots (see Figure 10 D and E), one of which corresponds to tryptamine. The lower spot in hexane chromatograms is divided on renewed chromatographing in *n*-hexane into two spots (Figure 10 F), whereas chromatographing in ethanol gives a spot in the position for tryptamine (Figure 10 G). These findings should be compared with the results for *f* (Figure 9 H, I, J, and K and L).

In an experiment synthetic tryptamine has been treated as the maize growth substance by shaking with phosphate buffer, pH 7.2. After acidification the solution was absorbed in kieselguhr, which was transferred to a glass column and eluted with ether. This was then fractionated, and the neutral fraction was examined. This gives on chromatographing in isopropanol—acetic acid-water or in ethanol spots with the same Rf values as the substance *f* from maize kernels, cf. Figure 10 H and I with Figure 9 B and C. On chromatographing this treated tryptamine in *n*-hexane, the substance acted like untreated tryptamine (cf. Figure 10 J and C). The position of the spots in the ethanol chromatograms varies, moreover, in different chromatograms of the treated tryptamine, as is the case with the position of the substance *f* in similar chromatograms.

Either tryptamine has been chemically altered during the extraction or substances which have followed along with the ether extraction and the subsequent fractionation have affected the substance during the course of the chromatographing. The latter seems to be the more probable. In one experiment, namely, ether was allowed to pass through a kieselguhr column acidified with the same amount of the phosphate buffer, pH 2.7, as in the experiments with maize. Thereafter the ether was shaken with alkaline glucose solution. If this ether is mixed with synthetic tryptamine and the mixture transferred to chromatograms, the results shown in Figure 10 K and L are obtained. In isopropanol—acetic acid-water one spot is obtained corresponding to untreated synthetic tryptamine and another corresponding to tryptamine treated as an extract of maize. The latter spot lies in the same place as the spot of the substance *f*.

Judging from the chromatographic experiments there is thus good evidence

Figure 10. Chromatograms of synthetic tryptamine in different solvents. A—G, untreated tryptamine; H—J, tryptamine treated as maize kernels (see text); K and L, tryptamine before chromatographing mixed with ether treated as maize kernels (see text). Further as in Figure 9.



that the substance *f* is identical with tryptamine. The only obvious difference is that tryptamine gives two spots on hexane chromatography, whereas the substance *f* does this on hexane chromatography first after it has been chromatographed in ethanol. This may be due to the occurrence of substances in the maize extract that prevent the decomposition of tryptamine.

The substance *g* is probably a decomposition product of tryptamine formed during the chromatographing. The substance *e* is closely related to the other two.

Tryptamine has earlier been isolated by White (1944) from stem tips and flowers of *Acacia*. In maize, on the other hand, it has not previously been demonstrated. Skoog (1937) found that *Avena* coleoptiles can convert tryptamine and tryptophane to auxin. Gordon and Nieva (1949) have shown that discs and enzyme preparations from pineapple leaves can transfer tryptamine to IAAld and IAA. According to Yamaki and Nakamura (1952), maize embryos also contain an enzyme which can form IAA from tryptamine. However Wildman, Ferri and Bonner (1947) have shown that spinach leaf tissue does not have the same ability. Even if tryptamine has been shown to be a possible intermediary product in the formation of IAAld and thereby IAA from tryptophane, it has, however, hitherto been considered more likely that IAAld and IAA are formed from tryptophane via IPyA, see Gordon (1954 and 1956) for further references to the literature.

The neutral fraction of ether extracts has also been examined for its content of growth regulators. In Figure 11 are presented histograms of double chromatograms in ethanol of such extracts. In these chromatograms

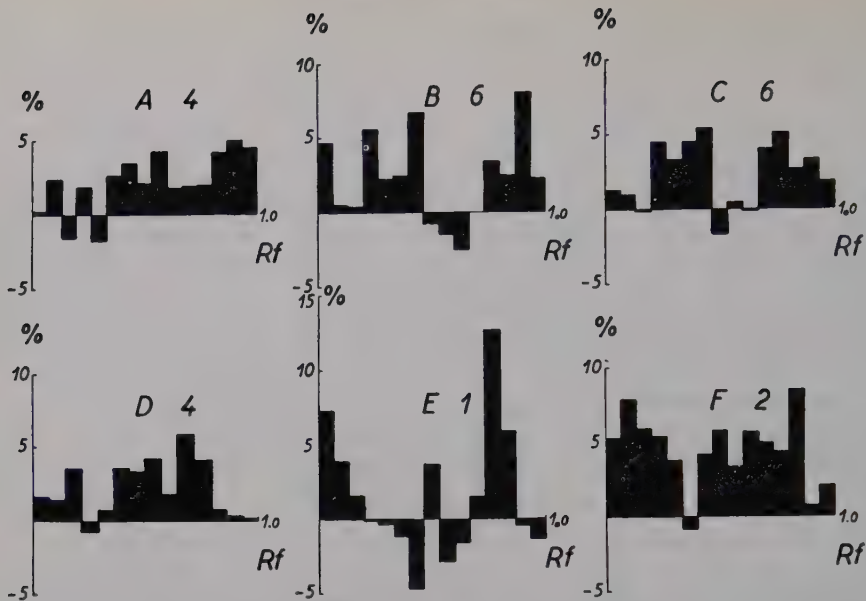


Figure 11. *Biological assay of chromatograms of neutral fraction of ether extracts from maize kernels (Hardy Canada Gold). A, B and C represent free auxin, D, E and F bound auxin. A and D represent dry kernels, B and E kernels swelled for 3 hours, C and F kernels swelled for 6 hours. The chromatograms were run in 70 % ethanol. Further as in figure 2.*

one cannot expect to find the substance *e*. The spot of application in the first chromatogram has been cut away before the rest of the chromatogram was eluted and subjected to a new chromatographing. A certain activity is found, however, in the spot of application and closest to this. The activity is probably due to the substance *g*, which, as earlier mentioned, is formed from tryptamine on chromatographing in ethanol. In addition to the substance *g* there are two stimulative regions in the chromatograms. Of these it is probably the one lying closest to the front which corresponds to tryptamine. Synthetic tryptamine obtained by dissolving the hydrochloride in NaOH and shaking this solution with ether, has about the same Rf value as this substance. Tryptamine obtained by ether extraction of maize should be expected to have the same Rf value as synthetic tryptamine, since the substance has not passed through kieselguhr. The other stimulative region, which lies between the spot of application and the solvent front, has the position that the eventually occurring IAAld should have. This substance, as previously stated, has been demonstrated in maize by Yamaki and Nakamura (1952). That the substance is not found in the phosphate buffer extracts is due either to its destruction during the extraction or its conversion into IAA.

As far as neutral inhibitors are concerned, these are largely masked by the stimulating substances. Especially *f*, which gives very long spots in ethanol chromatograms, can probably conceal inhibitors. In the chromatograms of ether extracts, where a suggestion of the presence of inhibitors has been detectable, these have been located halfway between the spot of application and the solvent front.

Supply of IAA to Swelling Maize Kernels

Hemberg (1955) has demonstrated that if dry maize kernels are allowed to lie for 3 hours in a solution containing 100 mg IAA per liter, these take up IAA. A large part of this is found immediately after the 3 hours as free auxin, whereas in kernels that have been allowed to lie in moist air for an additional 21 hours it is found chiefly as bound auxin. When these experiments were repeated, however, the earlier results were not completely reproducible. In the later experiments, which were performed with three different maize varieties, the kernels were examined for their content of free and bound auxin, partly immediately after they had lain for 3 hours in water or in IAA solution, partly after they had lain for an additional 3 hours and for an additional 21 hours in moist air. The results, which are presented in Figure 12, show that the increase in the free auxin induced by the IAA treatment is always greatest immediately after the treatment and falls markedly when the kernels are allowed to lie in moist air. On the other hand, the increase of bound acid auxin induced by the IAA treatment is already noticeable immediately after this, but it rises further in all experiments in the kernels that have been allowed to lie in moist air for 3 hours after the treatment and falls again in the samples that have been allowed to lie in moist air for 21 hours after the treatment. It is evident that IAA is taken up as free auxin, after that it is converted into bound auxin and later disappears from the kernels.

It has been shown by paper chromatography that the increased amount of free and bound acid auxin obtained on treatment of maize with IAA is due to the IAA (see Figure 12 D). This experiment has been carried out in the same manner as the earlier ones (Figure 12 A, B and C) but the acid fraction of the ether extracts has been subjected to chromatographing in isopropanol—ammonia-water and the chromatograms have been sprayed with Ehrlich's reagent. On this occasion distinct spots appeared in the position for IAA. After a day or so spots with lower R_f values could also be observed; it may possibly be a case of the substance x_4 demonstrated by Stove and Thimann (1954), which has been described in an earlier chapter of the present work as the substance

a. The spots for this substance, however, varied so greatly in intensity that no reproducible values could be obtained. The intensity of the IAA spots was measured in a photometer, and from these values the increase in the free and bound IAA induced by the IAA treatment was calculated. As is seen from Figure 12, D the values obtained correspond completely with those from the earlier biological determinations of free and bound acid auxin by means of IAA treatment. This shows clearly that IAA is taken up in the kernels and appears there as free IAA, which however rapidly disappears through conversion into a bound form. Also the bound IAA disappears rapidly from the kernels. Moreover some of the free IAA is probably destroyed by oxidation, cf. Galston (1956).

A similar experiment with IAET supplied to maize indicates that this is taken up by the kernels and is found both as free and bound IAET, but that some is converted into IAA which is also found in the free and bound form. IAET is, of course, found in the neutral fraction. This fraction contains, as previously stated, considerable fat. Therefore the neutral fraction prior to chromatographing has been purified according to Nitsch (1956) by shaking in acetonitrile solution with n-hexane, the latter substance dissolving the fat but not IAET. The purified neutral extracts have thereafter been chromatographed in n-hexane in air of 100 per cent humidity. The chromatograms have been sprayed with Ehrlich's reagent, and the amount of the different substances has been determined with a photometer.

If one wishes to determine to what extent IAA, when it begins to disappear from the maize kernels, is converted into some other substance, the neutral as well as the acid fraction should, of course, be examined. As earlier mentioned, the neutral fractions are rich in fat. A shaking of the substances in acetonitrile with n-hexane, however, can give certain losses. Therefore in the continued investigation the maize kernels have been extracted with phosphate buffer. The extracts have then been purified and fractionated in the previously described manner. All extracts of the neutral fraction contain the substance *e*.

In one experiment extracts of kernels which had been allowed to swell in water or in IAA solution were chromatographed in ethanol. Thereafter *e* was eluted and chromatographed in isopropanol—ammonia-water. The chromatograms were sprayed with Ehrlich's reagent and the amount of *e* determined photometrically. The substance *f* was also eluted from the ethanol chromatograms and chromatographed, but in isopropanol—acetic acid-water. After spraying with Ehrlich's reagent the amount of *f* was determined photometrically.

It is now evident (see Table 3) that in extracts from kernels which have been in IAA for 3 hours there is more of the substance *e* than in extracts of

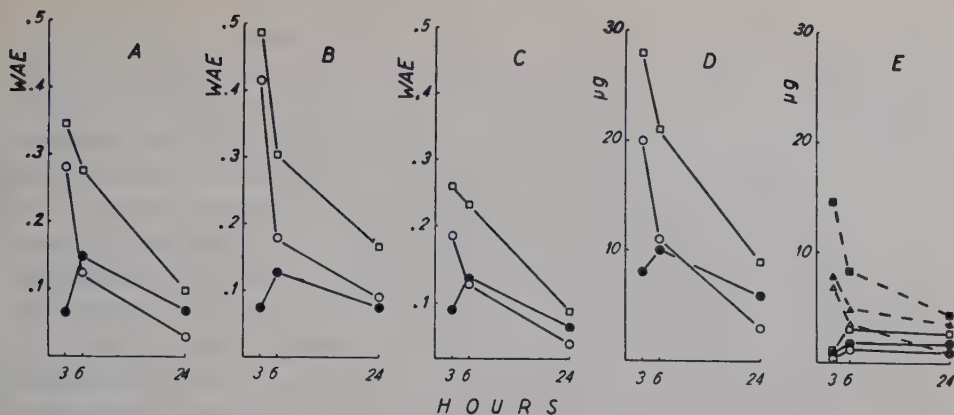


Figure 12. *Induced increase in amount of free and bound acid auxin and of free and bound IAA or IAet in ether extracts from maize kernels trough treatment with solutions of IAA (A, B, C and D) and IAet (E). A, Golden Rocket; B, Express; C and E, Hardy Canada Gold; D, Mette. ○ free auxin or free IAA, ● bound auxin or bound IAA, □ Total auxin or total IAA, △ free IAet, ▲ bound IAet, ■ total IAet. Abscissa: Swelling time in hours. Ordinate: Amount of free and bound auxin and of free and bound IAA or IAet in treated kernels after reduction of the amount in kernels treated with water. In A, B and C the amount is expressed in WAE per kernel, in D and E in µg IAA and IAet per kernel.*

kernels which have lain for the same length of time in water. The amount is further increased in extracts of kernels which have been allowed to swell in the air for 21 hours after the AA treatment. Kernels that have swelled for a total of 48 hours, on the other hand, contain about the same amount of *e* regardless of whether they have lain in IAA solution or in water. The values for *f* vary so greatly that nothing certain can be said as to whether the amount of *f* is increased or not by the IAA treatment.

It has been established, however, that in connection with the IAA treatment, when the amount of free and bound IAA disappears, there is a temporary increase in the amount of *e*. One may therefore assume that *e* is formed from IAA. As earlier stated, the amount of *e* is temporarily increased during the swelling, in connection with an increase in the kernels' natural content of free and bound auxin. Therefore *e* must be assumed to be a precursor of IAA.

Some experiments have been carried out to demonstrate the eventual occurrence of indoleacetylaspatic acid in maize kernels. On this occasion the technique of Andreae and Good (1955) was employed. The crushed kernels were treated with a bicarbonate solution, from which after acidification to pH 4.0 and shaking with ether the auxins was removed. The solution was thereafter acidified to pH 2.6 and shaken 3 times with *n*-butanol in order to obtain eventually occurring indoleacetylaspatic acid. Dry maize kernels were

examined and also kernels which after they had swelled in water or IAA solution (100 mg per liter) for 3 hours were allowed to swell in air of 100 per cent humidity for an additional 21 hours. In all butanol extracts on chromatographing in isopropanol—ammonia-water two weakly Ehrlich-positive regions were found, one in the spot of application and the other with the R_f value 0.06. This value corresponds well with that of indoleacetylaspatic acid (0.07). No difference in the colour intensity of the spots in chromatograms of IAA-treated maize and water-treated maize, however, could be demonstrated.

Experiments have been carried out to hydrolyze the butanol-soluble substance with the R_f value 0.06 found in maize by boiling with 2-N NaOH for 30 minutes. After the boiling the solution was acidified with HCl to pH 4.0 and shaken with ether, while the water phase was thereafter acidified to pH 2.6 and shaken with *n*-butanol. No IAA could be detected in chromatograms of the ether phase. In the butanol phase chromatographed in phenol-ammonia a ninhydrin-positive substance with the R_f value 0.8 was demonstrated. Since aspartic acid in phenol-ammonia has the R_f value 0.1, the substance demonstrated in the butanol cannot be aspartic acid. It is thus evident that indoleacetylaspatic acid does not occur in maize kernels. On the other hand, Good *et al.* (1956) have shown that it occurs in small amounts in maize coleoptiles that have been treated with IAA.

Discussion of the Results

From the results it is evident that all auxins in maize kernels demonstrated by biological assay occur both in the free and in the bound fraction. This should be explainable by assuming that all substances can occur partly in the free form and partly bound to some other substance, probably protein, from which they can be liberated on prolonged ether extraction. Spiegel and Galston (1953) and Galston (1956), as earlier mentioned, have demonstrated in any case that IAA can be bound to protein.

Andreae and Good (1955) and Andreae and Ysselstein (1956) have shown that pea seedlings can bind exogenously supplied IAA through the formation of indoleacetylaspatic acid. This formation, however, has not been demonstrable in maize kernels.

When a maize kernel is immature, the content of free auxin is high and the content of the bound still higher. With increasing maturation the content of both substances in the kernel falls considerably. This is probably due to the formation of some inactive substance. In the swelling of mature maize the content of bound auxin increases first. Thereafter free auxin is formed from this and consequently the amount of bound auxin decreases (Hemberg,

1955). The bound auxin which is formed during the swelling is probably derived from an inactive precursor. The intermediary link between this and the bound auxin is probably the neutral indole derivative *e*, still chemically unidentified. This is evident from the following facts. When maize kernels swell in water, the amount of *e* increases during the first hours and later decreases. IAA-treated maize contains during the first 24 hours of swelling considerably more of the substance *e* than the water-treated. In the IAA-treatment the kernels' content of free and bound IAA increases temporarily, and this increase in the IAA content of the kernels gives rise to the formation of *e*. The reaction thus proceeds in the opposite direction on the introduction of excess IAA.

In unripe maize at the milk stage there are found considerable amounts of free and bound auxin, but hardly any demonstrable amounts of *e*. At the early dough stage measurable amounts of *e* have already been formed, and ripe maize contains relatively large amounts.

Also the substance *f*, which has been demonstrated chromatographically to be identical with tryptamine, is probably an intermediary link in the conversion of IAA to inactive precursors, but the determinations of this substance have hitherto been impaired by so great experimental errors — *i.a.* due to the fact that the substance seems to be extremely unstable — that nothing definite can be said about the role played by *f*.

Summary

1. A method has been worked out with phosphate buffer, pH 7.2, for the extraction of auxins and growth inhibitors from maize kernels.

2. The content of free and bound acid auxin in maize kernels is greatest in unripe maize and decreases in connection with the maturation of the kernels.

3. Four different growth stimulators have been found in the acid fraction of ether extracts of maize kernels of different degrees of swelling. On chromatographing in isopropanol — ammonia — water these have the following Rf values: *a*, 0—0.1; *b*, 0.2—0.4 (IAA); *c*, 0.5—0.8 and *d*, 0.8—1.0. Inhibitors have been detected with the Rf values 0.1, 0.5 and 0.8—1.0.

4. Two growth-stimulating substances, *e* and *f*, have been found in the neutral fraction of phosphate buffer extracts. Their Rf values in different solvents have been determined (see Table 4). In chromatographing *f* forms partly another substance, *g*. *e* is easily converted into a mixture of *f* and *g*. All three substances exhibit a reaction with Ehrlich's reagent and therefore they all are probably indole compounds. They also react with ninhydrin. Paper chromatography has shown that *f* is identical with tryptamine (see

Figures 9 and 10). *e* and *g* have not been identified. *f* has also been found in ether extracts.

5. *e* apparently constitutes an intermediary link between IAA and inactive IAA precursors.

This investigation has been carried out partly with the aid of a grant from the Swedish Science Research Council. The author is indebted to Mrs. Maija-Liisa Holmberg for valuable technical assistance. The maize employed in this study has been supplied by A.B. Weibull, Landskrona, through the courtesy of Dr. O. Gelin. Dr. N. Å. Jönsson has synthesized the preparations of IAET and IAAM used in the investigation.

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The Production of Fruit Bodies in *Collybia velutipes*

II. Further Studies on the Influence of Different Culture Conditions

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In the first paper of this series (Aschan 1954) the influence of acid hydrolysed casein, tryptophan and thiamin was shortly mentioned. Further experiments, using liquid media, have now been made. As the effect of tryptophan might possibly be connected with its function as a precursor of indoleacetic acid, this latter substance was also included. The effects of variations in the glucose, the ammonium tartrate and the thiamin concentrations, and of different modes of pre-treatment of the cultures have also been studied.

Material and Methods

The dicaryotic *Collybia velutipes* strain L 1×L 7. was used in all experiments. The basic nutrient media used are:

Min (Aschan 1952;=*Min*₁, Aschan 1954): glucose-ammonium tartrate medium.

Max (Aschan 1952): *Min* with additions of yeast extract, malt extract and acid hydrolysed casein.

Pl (= *Pl*₀, Aschan 1954): sucrose-asparagine medium.

The pH was adjusted with NaOH or HCl (1-N). For added substances the following abbreviations are used: AM=ammonium tartrate, CAS=acid hydrolysed casein (Difco "casamino acids"), GLU=glucose, IAA=indoleacetic acid, THI=thiamin, TRY=tryptophan.

The cultures have either been used *directly* (*D*) after the inoculation, or they have been *pre-treated* (*P*), unless otherwise stated, during one week in darkness at +25°C, before the beginning of the experiment proper. The period of pre-treatment is always included in the reported length of the growth period. Unless otherwise mentioned,

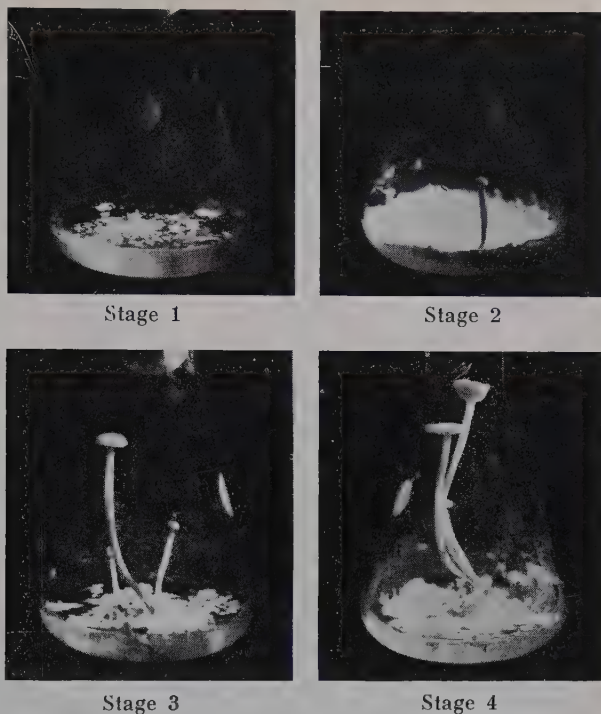


Figure 1. *The developmental stages 1—4.* (See text below).

125 ml Erlenmeyer flasks containing 25 ml nutrient solution have been used for the cultures. For further details see Aschan (1954). The cultures used for inoculation were, however, grown on 1.5 per cent agar with 2.5 per cent malt extract.

Quantitative comparison of fruit body production under different conditions presents great difficulties. In one series the fruit bodies may be small but fully expanded, while in another they are much larger but still immature. The variability between replicates of the same series is usually higher than for cultures in the vegetative condition. The number of fruit bodies produced cannot be deemed a good fruiting index (Plunkett 1953).

The best picture of the fruiting under different conditions is naturally obtained by repeated harvests after different periods of growth, recording the stages of development as well as the amount of dry matter produced. Due to the limited space in the temperature control chambers this has been done in one experiment only (Exp. 13). In the other cases the experiments were harvested a few days after the first fruit bodies had reached a fully mature stage. The developmental stage reached at this time in each flask has been judged from the most advanced of the fruit bodies and graded according to the following scale (*cf.* Figure 1):

- 0: mycelial growth only,
- 0.5: fruit body primordia observable as dark points but lacking visible stipes,
- 1: stipes slightly stretched,
- 2: stipe stretched, pileus conspicuous but not mature,
- 3: pileus mature (shedding spores) but still convex,
- 4: pileus flattened.

(The "rudimentary fruit bodies" mentioned in the paper of 1954 correspond to stage 1 and 2.)

For the characterization of the developmental stage reached in a series of replicates the average value of the numbers assigned to the individual flasks has been used (fruit body index or I_f).

The results of the experiments are usually shown graphically in the figures, where the details concerning the culture conditions will also be found. As the variability of the experimental data refers both to the dry matter production and to the developmental stage reached, an exclusive use of average values might easily become misleading and a rather full representation of the individual values has therefore been aimed at.

Experimental Results

Effects of different pre-treatments, of the pH of the medium and of different temperatures. Experiment 1 (Figure 2) in the present paper supplements the Min₁-series in Experiment 1, 1954. In the present study, however, harvesting was done at the same time for all treatments and replicates, and a larger number of the replicates therefore failed to produce fruit bodies at the conclusion of the experiment. In spite of this difference of procedure it is clear that the present experiment (15°C), contrary to previous experience, demonstrates that "pre-treatment" does not invariably delay fruit body production.

Such a delay has been observed in many experiments. The earlier fruit bodies in the "direct" cultures have, however, been rather small and they have sometimes, though not always, appeared in close connection with the pieces of mycelium used for inoculation. In order to test a possible effect of the conditions used for growing the inocula these were varied in the present Experiment 1. In all of the different treatments half of the number of replicates were inoculated with material from cultures kept under the daily rhythm of light and darkness at a temperature of about 18 to 20°C, while the material used for inoculation of the other replicates had, as usual, been grown at 25°C in darkness. The different types of inocula did not cause any significant difference during the following growth.

In Experiment 7 (Figures 4 and 5) the *a* series was placed directly in the greenhouse (D), while the *c* series was first placed in darkness at 25°C for one week (*P*). As both series were harvested a few days after the first fruit bodies had reached a fully mature stage it appears that the pre-treatment in this case delayed the fruit body production considerably, but having begun it was more profuse and uniform. Such greenhouse experiments could only be made during the winter months, when the temperature could be kept low enough for fruiting.

In Experiment 8 (Figure 6) the pre-treatment conditions were varied.

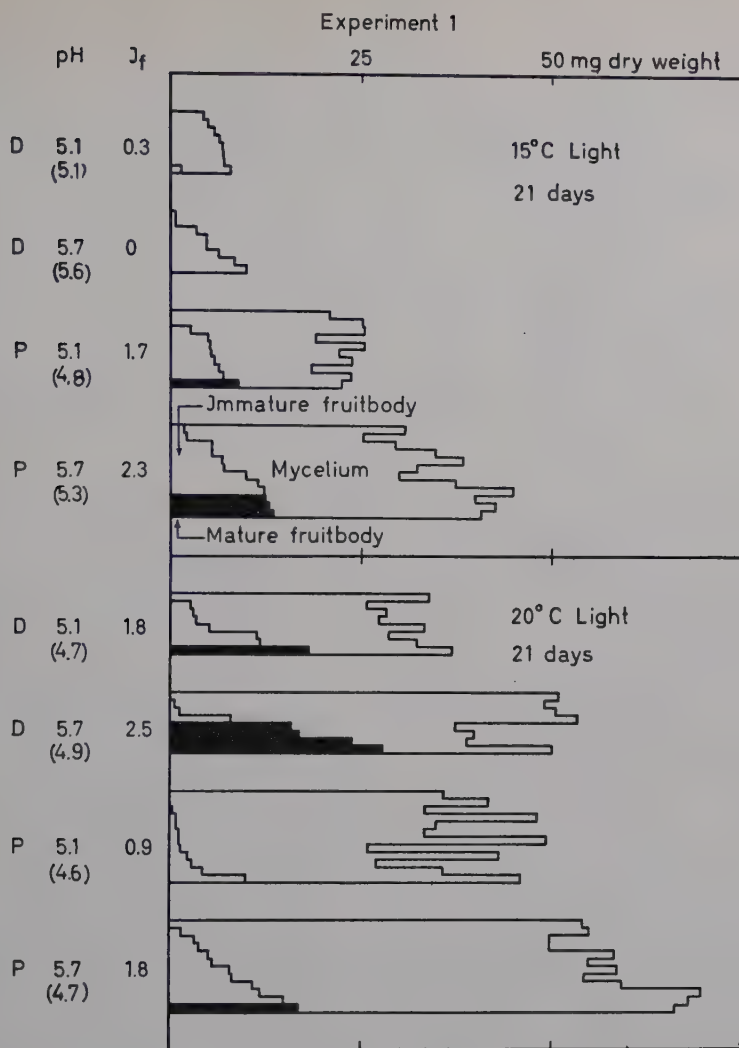


Figure 2. Dry matter production and fruiting of *Collybia velutipes* under different culture conditions. D "direct" and P "pre-treated" cultures (see p. 312) in solution in all cases. The pH value without parenthesis is the initial one and that within parenthesis is the final one. I_f = fruit body index (see p. 314). The dry matter of each replicate (8 to 12 under each condition) is given in the following manner: the low unfilled columns represent fruit bodies of stages 1 to 2, the filled columns fruit bodies of stages 3—4 (see Fig. 1), and the highest unfilled columns (measured from zero) the mycelium. To obtain the total dry matter production the sum of the lower and the higher columns (measured from zero) must be computed.

Table 1. *Synopsis of the effects of acid hydrolysed casein (CAS) on the fruiting of Collybia velutipes in Experiments 3 to 7. Min-solution, light. Gr: flasks kept in a greenhouse with fluctuating temperature (extremes +4° and +30°C). P and D see text p. 312.*

Exp.	Culture conditions			Fruit body index (I _f)			
	Temp.	Initial pH		CAS, g/l			
				0	0.05	0.5	2
3	15°	5.2	P	2.5	—	—	4
4a	10°	5.2	P	3	—	—	1
4b	15°	5.2	P	4	—	—	3.5
5	15°	5.8	P	3	—	2.8	0.7
6	Gr	5.8	D	0.5	0.4	1.6	1.0
7a	Gr	5.8	D	2.6	2.8	2.4	0.4
7b	15°	5.8	P	3.8	4.0	3.4	3.8
7c	Gr	5.8	P	4.0	4.0	3.9	3.2

During a pre-treatment in darkness the temperature must apparently be at least about 20°C and preferably 25°C in order to give a subsequent fruit body production characterized by high *I_f*-values and large dry weights. The influence of illumination during a pre-treatment at 25°C is slight and probably unfavourable.

Before entering upon the discussion of the pH effects obtained in the present study, some modification will be made of the description of Experiment 5 in Aschan (1954), which is unclear due to misprints. The first harvest was made after 24 days as stated in the figure (not after 34 days as stated in the text). A second harvest was made after 63 days, comprising the cultures kept at 15°C in darkness and also a series, not recorded in the figure, which was kept at 20°C in light and included three replicates at each pH value. In this latter series mature fruit bodies had been produced in all replicates on all media with initial pH values between 4.0 and 7.3, and the optimum was apparently in the range 5.1—6.1 in spite of the increased earliness at pH 4.0 observed at the first harvest (after 24 days). No dry weight determinations were made, however, because of the overripeness of the fruit bodies. In the present Experiment 1 (Figure 2) an initial pH value of 5.7 has been more favourable for the onset of the fruit body production than an initial value of 5.1. Some further support for the supposition that an initial pH value of about 5.7 might be more favourable for fruit body production than somewhat lower values is given by Experiment 1 in Aschan (1954), where initial pH values of 5.2—5.3 delayed fruit body production on the *Min* medium at 20°C.

In the present Experiment 1 the fruit body production is as good at 20°C as at 15°C. Such cases have been reported also in the earlier paper (Aschan 1954) but, as pointed out there, the higher temperature is probably close

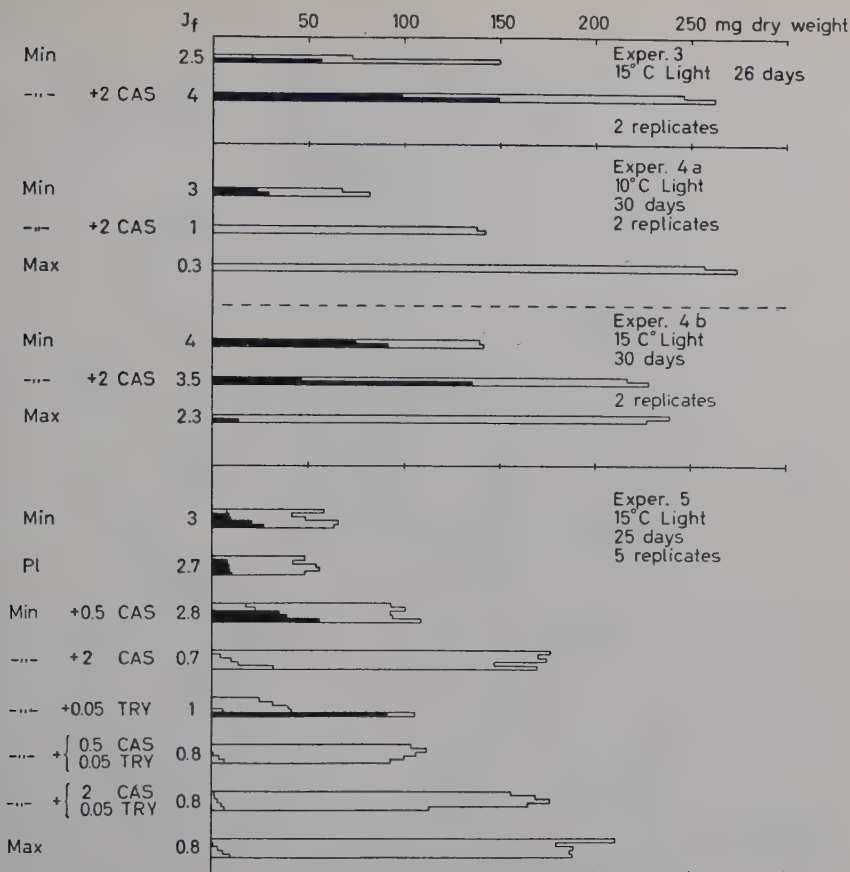


Figure 3. Influence of acid hydrolysed casein (CAS) and tryptophan (TRY) on the fruiting of *Collybia velutipes*. Data presented as in Fig. 2. Pre-treatment (P). The concentration of CAS and TRY in gram per litre. Culture solutions: Min, Max and PI (See p. 312).

Table 2. Synopsis of the effect of tryptophan (Try) on the fruiting of *Collybia velutipes* in Experiment 5—7 a. pH 5.8, light. Gr, P and D as in Table 1.

Exp.	Culture conditions			Fruit body index (I_f)			
	Temp.	Medium		Tryptophan g/l			
				0	0.0005	0.005	0.05
5	15°	Min	P	3	—	—	1
5	15°	Min + 0.5 CAS	P	2.8	—	—	0.8
5	15°	Min + 2 CAS	P	0.7	—	—	0.8
6	Gr	Min	D	0.5	2	1	0.2
6	Gr	Min + 0.5 CAS	D	1.6	—	1.5	1.4
7a	Gr	Min	D	2.6	1.8	2.6	1.8
7a	Gr	Min + 0.5 CAS	D	2.4	—	2.1	0.8

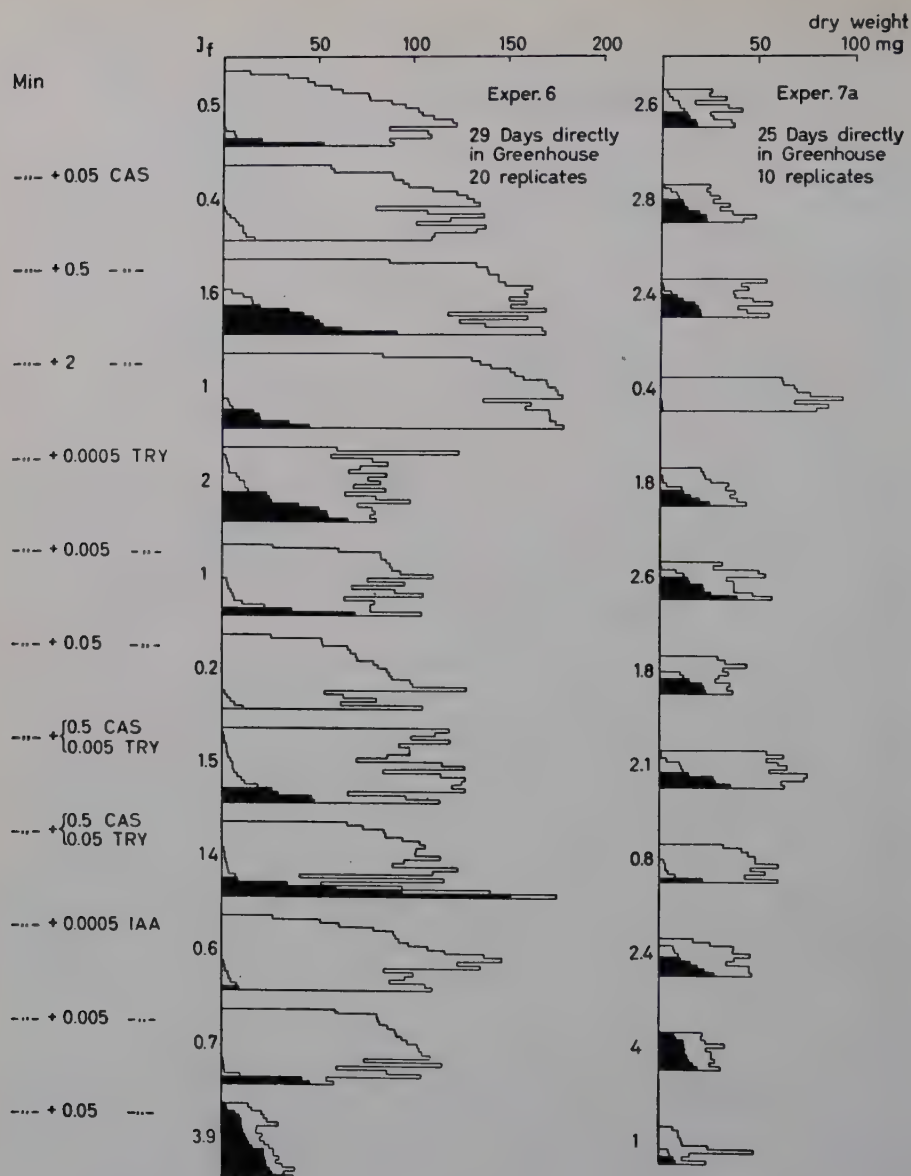


Figure 4. Influence of CAS, TRY, and IAA additions on the fruiting of *Collybia velutipes*. Data presented as in Fig. 2. Concentrations in g. per l.

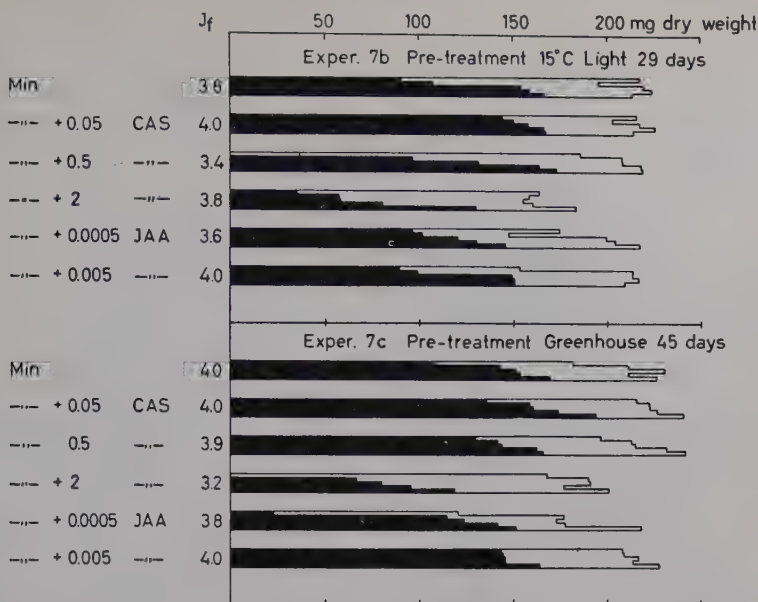


Figure 5. Influence of CAS and IAA on the fruiting of *Collybia velutipes*. Data presented as in Fig. 2. Concentrations in g. per l.

to the upper limit for fruit body production of the strain used, and may often lead to total failure of such production. In the following experiments a temperature of 15°C has therefore usually been preferred.

The data obtained in Experiment 4 (Figure 3) confirm the earlier results (Aschan 1954) which showed that fruit body production is earlier at +15°C than at +10°C.

Comparison between shaded and unshaded cultures (Experiment 2). Earlier observations (Aschan 1954) on agar media showed a tendency to better fruit body production on slightly shaded tubes than on unshaded. An experiment was therefore performed with liquid *Min* substrate (initial pH 5.1; pre-treat-

Table 3. Synopsis of the effect of indoleacetic acid (IAA) on the fruiting of *Collybia velutipes* in Experiment 6—7 *Min*-medium, pH 5.8, light, *Gr*, *P* and *D* as in Table 1.

Exp.	Culture conditions		Fruit body index (<i>I_r</i>)			
	Temp.		IAA, g/l			
			0	0.0005	0.005	0.05
6	Gr	<i>D</i>	0.5	0.6	0.7	3.9
7a	Gr	<i>D</i>	2.6	2.4	4.0	1.0
7b	15°	<i>P</i>	3.8	3.6	4.0	—
7c	Gr	<i>P</i>	4.0	3.8	4.0	—

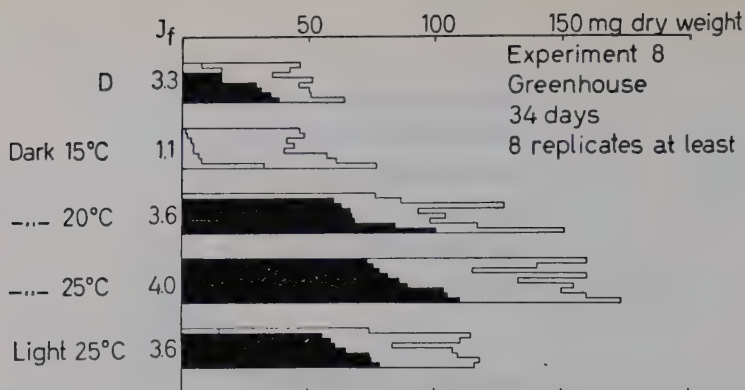


Figure 6. The effect of different types of pre-treatment on the fruit body production of *Collybia velutipes*. Min solution. Initial pH 5.8. D: cultures placed directly in the greenhouse. The other cultures have been pre-treated one week under the conditions given to the left of the diagram. Data presented as in Figure 2.

ment; final growth at 10°C) where 16 flasks were partly covered with dark paper in such way that only diffuse light reached the fungus. The same number of flasks were unshaded. The experiment was followed during 36 days. No dry weight determinations were made but from the I_f values (shaded series: 1.8, unshaded: 2.4) it is clear that the shading had no positive effect on the fruiting. As the full light intensity was the same as in the earlier experiments it seems probable the opposite tendency observed in them was fortuitous.

Influence of acid hydrolysed casein (CAS). In the previous paper (Aschan 1954) a stimulating effect of quite low CAS concentrations in the agar medium was reported. Further experiments (No. 3 to 7) with liquid media show, however, that this effect is not easily reproduced (see Figures 3, 4, and 5 and Table 1). Experiments 3 and 4 were of a preliminary nature and the differences may, due to the low number of replicates, be fortuitous. In Experiment 5 and 7 the fruiting was good in the control, and the CAS additions had only detrimental effects on fruit body production. For Experiments 5, 7 a and 7 c (Table 1) this is apparent from the I_f indices, while in Experiment 7 b the negative effect is clear from the decreasing dry weight of the fruit bodies (Figure 5). Furthermore, observations made during the growth period indicated no hastening of fruiting.

Only in Experiment 6 (Figure 4) was the fruiting tendency of the control cultures rather low. This may be related to an unintentionally long autoclaving of the media, which caused them to acquire a darker brown colouration than usual, and which is the only factor differing in Experiment 6 and

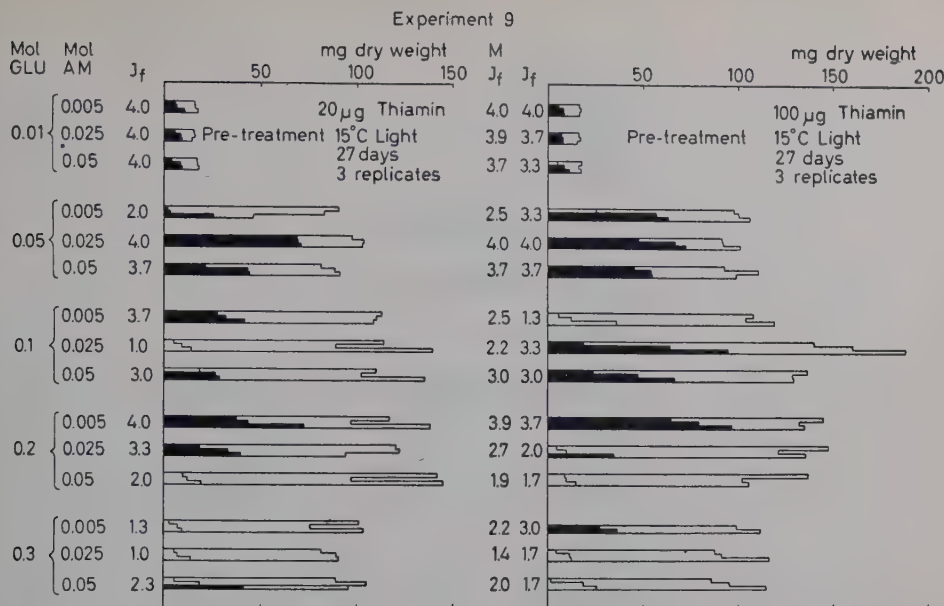


Figure 7. Effect of varying glucose (GLU) and ammonium tartrate (AM) concentrations on the fruiting of *Collybia velutipes*. Min solution with altered AM, GLU and thiamin concentrations. Initial pH 5.8. Data presented as in Fig. 2. $M I_f$: average value for both series at different thiamin concentrations.

7 a. The media were not unfavourable for growth in general, however, as is apparent from the rather high production of mycelial matter. Under the circumstances there is an unmistakeable stimulation of the fruit body production at a concentration of 500 mg CAS per liter, a concentration which is, however, much higher than the optimum found for agar cultures (Aschan 1954).

It is noteworthy that the addition of CAS to the *Min* medium has generally stimulated the mycelial growth, which is also higher on the *Max* than on the *Min* medium.

Influence of tryptophan (TRY). In Experiment 5, 6, and 7 a the effect of varying tryptophan concentrations have been tested. From the synopsis of the I_f -values in Table 2 it is clear the highest TRY concentration (0.05 g/l) has always tended to depress the fruiting. As in the case of the casamino acids a stimulating effect is apparent only in Experiment 6, where the fruiting of the control is relatively low; in the presence of a stimulating CAS concentration the tryptophan effect disappears. In Experiment 7 a it would seem that the inhibiting effects of high CAS and TRY concentrations are added.

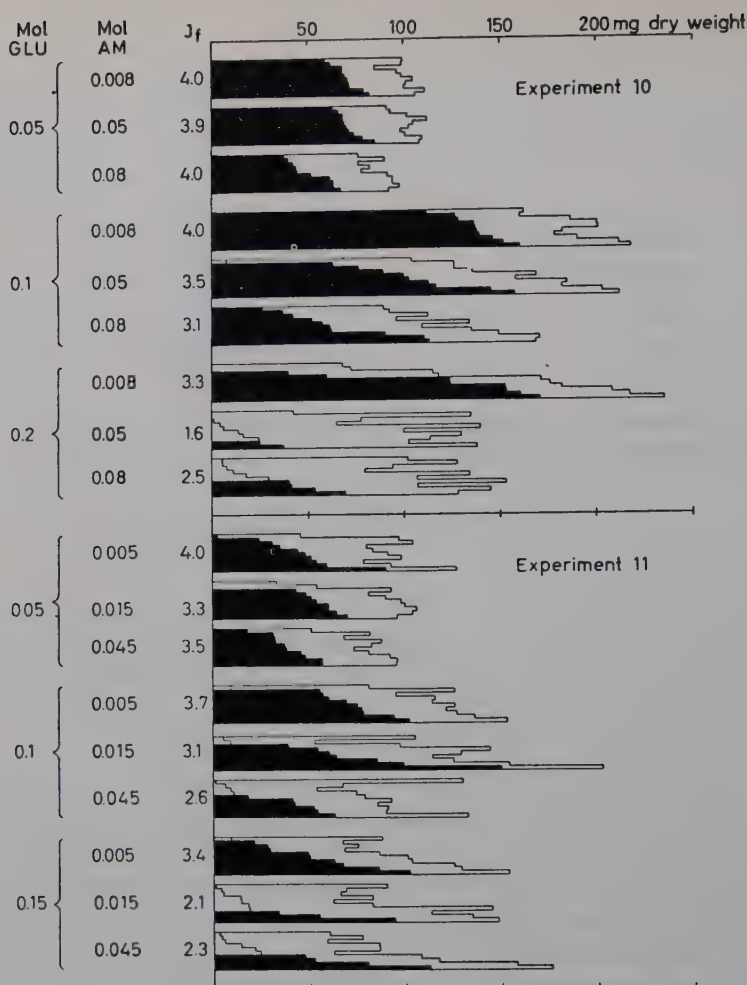


Figure 8. Effect of different glucose (GLU) and ammonium tartrate (AM) concentrations on the fruiting of *Collybia velutipes*. Min solution with altered GLU and AM concentrations. Initial pH 5.8. Culture conditions: P, 15°C, light. Harvest after 28 days. Data presented as in Fig. 2. 10 replicates.

Influence of indoleacetic acid (IAA). Like the casamino acids and tryptophan IAA may under favourable circumstances stimulate the fruiting (see Figures 4 and 5 and Table 3). In Experiment 6 the stimulation coincides with the appearance of a conspicuous reduction of the total dry matter production at 0.05 g/l IAA. It would seem, however, that earlier fruiting is not directly connected with reduced dry matter accumulation, as in Experiment 7 a stimulation is indicated at a tenfold lower concentration which is not signi-

Experiment 12

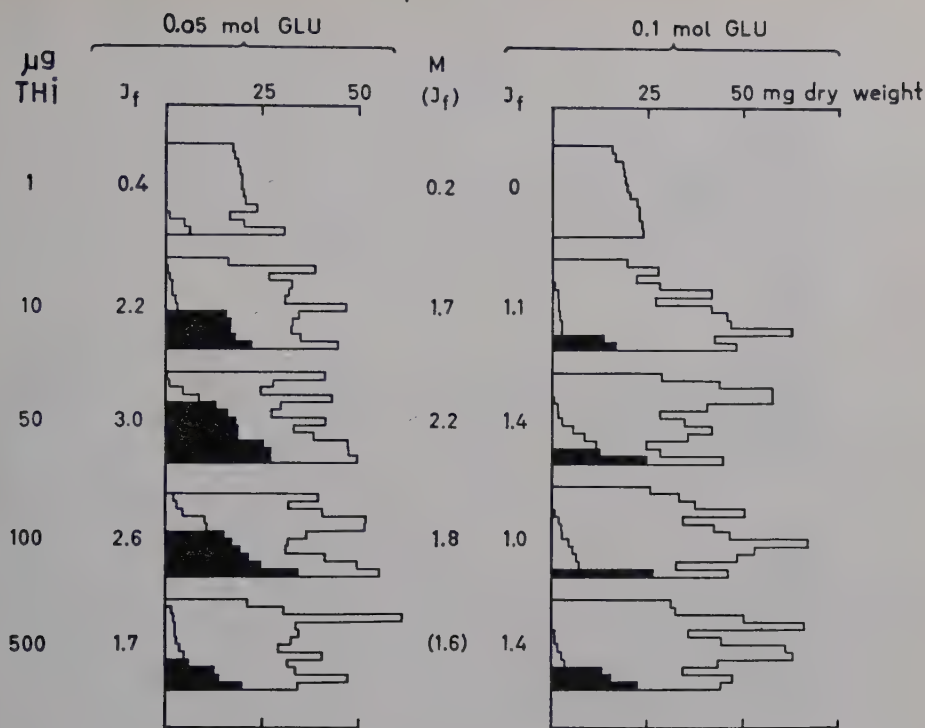


Figure 9. Effect of varied thiamin (THI) and glucose (GLU) concentrations on the fruiting of *Collybia velutipes*. Min solution with altered GLU and THI conc. Initial pH 5.8. Culture conditions: P; +15°C, light. Harvest after 28 days. Data presented as in Fig. 2.

ificantly inhibiting for the dry matter production. In Experiments 7 b and 7 c the fruiting was optimal already in the control cultures, and no effect of non-inhibiting concentrations of IAA was found.

Effects of different glucose, ammonium tartrate, and thiamin concentrations. In Experiment 9 (Figure 7) a wide range of glucose concentrations were used and the ammonium tartrate concentration was also varied. Taking into account the results obtained by Hawker (1942), which indicate that *Collybia velutipes* and many other basidiomycetes as well as ascomycetes need a higher glucose concentration at higher thiamin levels in order to maintain optimal conditions for fruit body production, the experiment was performed at two different thiamin concentrations (20 and 100 µg). No clear interaction between the glucose and thiamin factors was, however, observed in the present experiment (see also Exp. 12 Figure 9), which is in good agreement with the earlier results obtained with agar media (Aschan 1954). The differ-

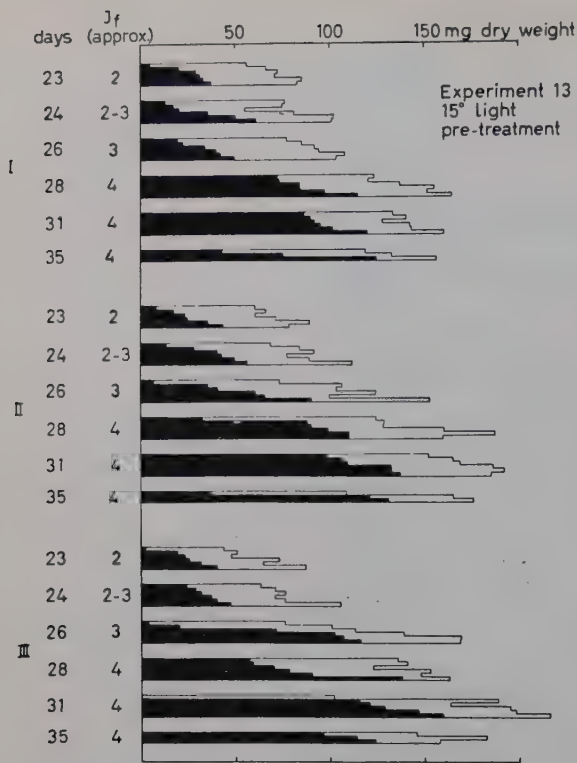


Figure 10. Effect of varying ammonium tartrate concentrations (AM) on the fruiting of *Collybia velutipes* at different harvest times Min solution but with GLU conc. 0.1 mol and the AM conc. 0.005, 0.01 and 0.15 in series I, II and III resp. Initial pH 5.8. Data presented as in Fig. 2, 6 replicates in each harvest except in the last one with only 3. Initiation of primordia (=stage 0.5 see text p. 313) observed after 17 days in most flasks.

ence between the present results and those obtained by Hawker may be due to the different strains used, or, and perhaps more likely, to differences in the composition of the culture media.

Nor for the glucose and ammonium tartrate factors can any clear interaction be detected in the present experiment. If we take the average of all I_f values for each glucose level, we obtain the following series corresponding to rising glucose concentration: 3.9 → 3.4 → 2.6 → 2.8 → 1.9, showing a conspicuous tendency to a decrease in the rapidity of fruit body development. The depressing effect of rising glucose concentration upon the I_f -values is accompanied at first by a sharp rise in the total dry matter production, which is finally followed by a slight decline at the highest glucose concentration.

In Experiments 10 and 11 (Figure 8) the medium range of glucose levels was further studied, and the fall of the average I_f -values with rising glucose concentration was again apparent (Exp. 10: 4.0 → 3.5 → 2.5; Exp. 11: 3.6 → 3.1 → 2.6). In these experiments there is also a fairly clear tendency, most pronounced at the highest glucose levels, for I_f -values to fall with increasing ammonium tartrate concentration.

In Experiment 12 (Figure 9) the effects of a wide range of thiamin concentrations were studied at two glucose levels. The depressing effect of rising glucose concentration on the I_f -values is very pronounced, and is not reversed even at the highest thiamin concentrations used (cf. p. 323).

In order to obtain further information on the effects of the ammonium tartrate concentration Experiment 13 was performed. The development was followed by repeated harvests but in spite of this no clear effect of the ammonium tartrate concentrations was apparent. The results may, however, be of some interest as illustrating the rate of fruit body production and maturation.

Discussion

Before entering upon a discussion of the results obtained in the present paper the bearing of the I_f -values used as an index of the fruiting conditions must be restated. They express the degree of maturation of the fruit bodies at the time of harvest. Observations made during the experiments show, however, that an early maturation corresponds to an early initiation of primordia (cf. also Plunkett 1953) and, therefore, high I_f -values also indicate pronounced earliness of the fruiting.

The present results clearly show a depressing effect of increasing glucose concentration upon the I_f -values which is the result of a delayed initiation of the fruit body production. The same result was obtained for increasing sucrose concentrations by Plunkett (1953) in his experiments with *Collybia velutipes*, and for glucose by Madelin (1956) in experiments with *Coprinus lagopus*. Hawker (1939) showed that glucose became inhibiting for the fructification of *Melanospora destruens* (measured as the number of perithecia formed) at a rather low concentration (1.0 g/100 ml), and the same result has been obtained for other ascomycetes with glucose (Hawker and Chaudhuri 1946). Sucrose, however, up to rather high concentrations (10.0 g/100 ml), raises the number of perithecia in *Melanospora*.

For many fungi a depressing effect on fruiting may be observed already at quite low concentrations of nitrogen supply (see Hawker 1957 p. 58). In the present experiments a slight effect of this type was obtained with rising ammonium tartrate concentration, but more conspicuous effects have been obtained earlier with asparagine (Plunkett 1953, Aschan 1954) or ammonium chloride (Aschan 1954) as nitrogen sources. Reports of the influence of different nitrogen sources on the fructification of other hymenomycetes are relatively few. Bille-Hansen (1953) showed that asparagine in a concentration of 4.5 g per litre was able to suppress the fruit body formation of *Coprinus sassii* on a medium with nitrate as a basal nitrogen source. Madelin (1956)

compared the effect of various nitrogen sources at a level equivalent to 0.033 *M* sodium nitrate on the fruiting of *Coprinus lagopus*. Ammonium chloride, ammonium nitrate, and alanine gave fruit body formation, but ammonium tartrate, urea, glycine, and asparagine inhibited it. For the same species Scheler-Correns (1957) observed fruit body formation on, among other things, asparagine and glycine. The concentration was, however, much lower (eq. to 0.006 *M* nitrate) than that used by Madelin and the importance of this factor is thus again apparent.

The effect of a certain level of nitrogen supply may be influenced not only by the nature of the nitrogen source, but also by the level of the simultaneous carbon supply. Madelin (1956) observed an interaction effect on the fruiting of *Coprinus lagopus* between glucose and alanine concentrations, and Plunkett (1953) obtained the highest fruit body yield of *Collybia velutipes* for the combination of low asparagine with high sucrose concentration.

In the present investigation no clear interaction between the nitrogen and carbohydrate factors have been found. In Experiments 10 and 11 there seems to be a tendency for the nitrogen inhibition of the fruiting to become stronger at higher glucose concentrations, which is at variance with the results referred to above. A close comparison is, however, impossible due to differences in the experimental techniques used.

It is a general rule that the environmental conditions favourable for vegetative growth of fungi may vary within wider limits than those leading to fructification (Hawker 1957). For the mycelial growth of *Collybia velutipes* Humphrey and Siggers (1933) found a temperature optimum at 24°C, which is in close agreement with results obtained for the present strain of this fungus by Aschan-Åberg (1956). With increasing length of the growth period the optimum tends to change to a somewhat lower temperature. For fruit body production, however, 24°C is decidedly supraoptimal or even completely inhibiting (Brodie 1936, Aschan 1954, Takemaru 1954). The upper limit seems to vary with the strain used. Plunkett (1953) thus obtained good fruit body production at 20°C, while Aschan (1954) only rarely observed fruiting at this temperature and found 15°C to be closer to the optimum. Takemaru (1954) recommends keeping the cultures at 5 to 10°C in order to obtain fruit body production.

The production of fruit bodies in fungi may be preceded by an amassing of reserve materials in the mycelium (*cp.* Hawker 1957 p. 49), and the beneficial effect of a period of pre-treatment at comparatively high temperature upon the fruiting of *Collybia velutipes* may be seen from this point of view. Too long pre-treatment periods, and also very rich nutrient solutions (*Max* medium, *Min* medium with high additions of *Cas* or glucose), on the other hand, might be supposed to give a vegetative growth of such strength that

the medium is changed in a way which prevents fruiting (pH changes, accumulation of toxic staling products, cp. Hawker 1957 p. 51). Plunkett (1953) also failed to obtain fruit body production on media containing large additions of yeast or malt extract.

The stimulating effect of tryptophan and indoleacetic acid on the fruiting of *Collybia velutipes*, which was obtained in some experiments of the present study is not easily explained. Further studies must decide, whether conditions can be found which lead to a regular appearance of these effects, and whether the tryptophan effects are possibly indirect and mediated by indoleacetic acid.

Summary

The effects of some external factors upon the fruit body formation (determined as I_f -values — see p. 314 — and dry weight) of *Collybia velutipes* on synthetic substrates have been studied. Pre-treatment during one week at 25°C in darkness, followed by culture at 15°C in light results in good and uniform fruiting. The initial pH of the medium should preferably be about 5.7. Additions of acid hydrolysed casein, tryptophan and indoleacetic acid stimulated fruiting in some experiments but not in others. Rising glucose concentration retards the fruiting, and the same tendency is in some cases apparent for rising ammonium tartrate concentration. No interaction between glucose and thiamin could be found.

This investigation has been supported by grants from the Swedish Natural Science Research Council and from Consul G. C. Faxé's Foundation.

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The Stimulation of the Respiration of Seeds with Gibberellic Acid and its Analytical Application

By

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It is already known that gibberellins influence the germination of seeds and also the respiration of plants (see Stowe and Yamaki 1957). In this paper we describe investigation on the influence of gibberellic acid on the respiration of different seeds. One of the reasons for this investigation was the possibility that a suitable method for the determination of gibberellic acid (and other gibberellins) could be worked out in this way.

The seeds investigated were seeds of barley, wheat, timothy, rape, and peas. One hundred grams of seeds (fresh weight) were soaked for 48 hours in 150 ml. of distilled water or in solutions of gibberellic acid¹ at different concentrations (1, 3, 10, 30, and 100 ppm.). This was done by placing thin layers of seeds in large petri dishes in order to secure an adequate supply of air. The petri dishes were kept in the dark at 25°C. After soaking, the seeds were transferred to glass containers (25°C) through which CO₂-free air was blown. The respiratory carbon dioxide formed was absorbed in soda lime in the usual way. The production of carbon dioxide was measured at different times up to 50 hours, calculated from the end of the soaking. From each measurement the carbon dioxide formation during one hour was calculated.

For all the seeds investigated it was found that the respiration was stimulated by the treatment with gibberellic acid. In the case of the rape seeds the stimulation was relatively slight; the seeds treated with a 10 ppm. solution of gibberellic acid showed an increase in respiration amounting to about 10 per cent. With all the other seeds the stimulation was greater, especially for the seeds of barley and wheat. Barley seeds treated with a 10 ppm.

¹ Kindly supplied by Merck-Sharp and Dohme, Research Laboratories, U.S.A.

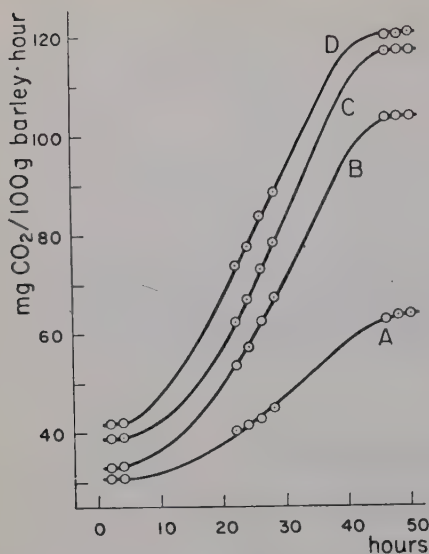


Fig. 1.

Figure 1. *Respiration of 100 g barley (fresh weight) after treatment with different concentrations of gibberellic acid.*

A: control without gibberellic acid.

B: 1 ppm. gibberellic acid.

C: 3 ppm. gibberellic acid.

D: 10 ppm. gibberellic acid.

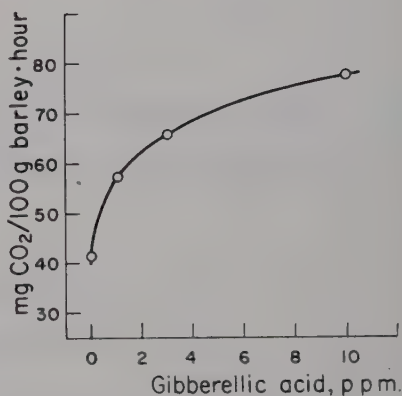


Fig. 2.

Figure 2. *Respiration of 100 g barley (fresh weight) after 24 hours.*

solution of gibberellic acid gave a respiratory value after 24 hours that was twice as high as the control. Even a 1 ppm. solution gave an evident stimulation. Solutions containing 10, 30, and 100 ppm. were found to give almost the same results.

Figure 1 gives some values obtained with barley. Each value represents the mean of 4 to 6 determinations. Only the values for 1, 3, and 10 ppm. are given as the higher concentrations did not show any additional stimulatory effect. Shortly after the seeds had been transferred to the containers, *i.e.* after 4 hours, the respiration is stronger in the seed treated with gibberellic acid and this increase in the respiration continued to the end of the experiment, at 50 hours.

In Figure 2 the respiration is given 24 hours after the soaking with different concentrations of gibberellic acid. Between 0 and 3 ppm. gibberellic acid a slight increase in the concentration of gibberellic acid causes a strong response in the intensity of the respiration, whereas the response is slighter at higher

concentrations. For analytical purposes thus concentrations below 3 ppm. are most suitable.

It may be mentioned that the uptake of water during the soaking is not influenced by the treatment with gibberellic acid.

Conclusions

Experiments, some of which are presented here, make it probable that the respiration of seeds may be used as a method for the quantitative analysis of gibberellins. The absolute values for the respiration are somewhat varying. However, the ratio between the respiration with and without gibberellic acid is relatively constant, provided that low concentrations are used. Barley seeds seem to be particularly suitable. A method, based on the principle proposed, has the advantage that the analysis can be completed in 3 days which is a short time compared to existing biological methods. The amounts of gibberellic acid which can be tested with this method are small, about 0.5—3 microgram per ml. solution. Further investigations concerning this method of analysis are in progress.

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Investigations on the Mechanism of Absorption and Accumulation of Salts I.

Initial Absorption and Continued Accumulation of Potassium Chloride by Wheat Roots

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(Received January 16, 1958)

1. Introduction

The mechanisms of absorption, accumulation, and transport of inorganic salts are still lingering as a challenging topic of experimental plant physiology. For a detailed presentation of the huge complex of problems dealing with salt absorption the reader is referred to a number of recent reviews (Lundegårdh 1954 b, 1955, 1958). The fundamental question, *viz.* the existence of quantitative relations between the accumulation of salts and one fraction of the respiration, the anion respiration (AR), was first introduced by Lundegårdh and Burström (1933). It has in the past years been repeatedly reinvestigated in this laboratory. The discovery of quantitative relations between salt absorption and anion respiration was followed by an approach into biochemistry, inasmuch as Lundegårdh and Burström (1935) inferred the cooperation of active iron in the anion respiration. Later on incisive spectrophotometric investigations of living roots proved the close relation between anion respiration and the oxidation reduction equilibrium of the cytochrome system (Lundegårdh 1951, 1952, 1954a). The end-place of salt accumulation are the sap spaces. Because accumulation of salts in the vacuoles means an increase in osmotic energy, and osmotic energy is capable of performing mechanical work, the anion respiration is one of the few examples of mecha-

nical work guided by the operation of a definite enzyme system. The performance of osmotic work is also reflected in the active transport of salts through tissues and whole plants (Lundegårdh 1943, 1945, 1950a).

Besides of these briefly stated facts concerning the biochemical background of active salt accumulation it was shown at an early date (Lundegårdh 1932, Lundegårdh, Burström and Rennerfelt 1932) that roots absorb cations and exchange cations much in the same way as inorganic colloids do. It was later shown that this absorption proceeds partly independently of active respiration (see Lundegårdh 1958). It was concluded at an early date, too, that non-metabolic absorption precedes the active accumulation (Lundegårdh 1940, Robertson 1944). Studies in the electrochemistry of the root surface (Lundegårdh 1938, 1940, 1941) pointed to the existence in the surface of the protoplasm of carriers R^+ and R^- absorbing cations and anions according to an ion exchange steered by the Donnan equilibrium. It was assumed that carriers in the bulk of the protoplasm are taking over salt ions by means of progressive exchange (Lundegårdh 1940, 1958), but the fairly wide extension of this type of absorption, not only of cations but also of anions remained unsettled until Hope (1953), Hylmö (1953) and others put forward the idea of an "apparent free space" (*cf.* Briggs and Robertson 1957), *viz.* a measurable capacity of the cells to absorb complete salts independently of active processes. The authors introducing the concept "free space", however, overlooked my earlier results (Lundegårdh 1943, 1945, 1950a) of a free non-metabolic flow of salts through the cortex of the roots. These investigations showed that cations *and* anions of mineral salts, which have been brought into the cortex by active anion respiration, are capable of moving into the central vessels and channels without the aid of respiratory processes which may be inhibited by cyanide, azide, or monoiodine acetate (Lundegårdh 1949b, 1950a). It was shown, however, that the velocity of the transport is enhanced by simultaneous anion respiration.

I have criticized the conception "free space" because it is too ambiguous, primarily because no distinction is made between diffusion and adsorption and/or ion exchange. The review of Briggs and Robertson (1957) overlooks several earlier results from this laboratory and does not touch the very important problem of a possible interference of respiratory processes in the initial phase of salt absorption. This problem is the main topic of the present investigation.

2. Technique

Spring wheat was sown on grids of stainless steel suspended near the surface of 4 lit. nutrient solution in glass troughs $15 \times 20 \times 20$ cm. The plants were illuminated by strip lamps at $15-20^\circ\text{C}$. The unbranched ends of the roots (ca. 60 mm.) were used

for the experiments. Each experiment was preceded by soaking the roots 2 days in distilled water.

Salt absorption and respiration were determined in Pyrex or Quickfit flasks with ground stoppers, completely filled with aerated solution. Quantities: 1—4 g. roots in 25—50 ml. solution. The relation between roots and solution is chosen with regard to an analytically convenient decrease of salt concentration and oxygen content during the experiment. It is important to rinse the roots with the test solution a few seconds before starting the experiment, in order to avoid undue dilution from distilled water adhering to the roots.

Chloride was determined by electrometric titration with 0.01 *M* AgNO₃ with phthalate at pH 3.2 as reference and AgCl-Ag-AgCl electrodes. If KCN is present the cyanide has to be removed by boiling in dilute acetic acid in order to avoid disturbance from AgCN. Potassium was determined by the Lundegårdh method of flame spectroscopy (Lundegårdh 1929—1934). The consumption of oxygen in the respiration — of which always both ground respiration and anion respiration were separately measured — was determined by the Winkler method. Determinations were also made by means of the Warburg respirometer, but a serious drawback is here the fact that wheat roots give off volatile substances which disturb the measurements. Further with the Warburg method salt absorption and respiration cannot be simultaneously determined. This is, however, possible with the Winkler method (see Lundegårdh 1949 c).

The vessels were vigorously shaken or rotated during the experiments. A water bath secured constant temperature, from +25° down to +1°C. Experiments were also performed with whole plants, the roots of which dipped in test tubes with salt solution. No difference in the salt absorption was observed in these experiments at a time of experiment of 2 hours. For more extended experiments, up to 5 hours, the solutions were changed in shorter intervals. The following figures show that in experiments lasting 2 hours quantities lost by bleeding (see Lundegårdh 1945, 1950 a) are negligible:

Accumulation from 0.005 *M* KCl at 20°C., calculated per h. and g. fr. wt.:

Whole plants and changed solutions	4.50 μ mol
Whole plants and stationary solutions	4.37 „
Cut roots and changed solutions	4.40 „
Cut roots and stationary solutions	4.10 „

3. The Time Course of Absorption and Accumulation

As shown in table 1 the times are chosen with regard to both an initial period and a continued accumulation. In these experiments, which were always run in four parallels, the start was made from a large set of vessels of which four were taken for analysis at the indicated times. Table 1 shows that both chloride and potassium are very rapidly absorbed during the first five minutes. The velocity here amounts to several times higher values than those observed in the period 30—60 minutes, in which the approximately constant accumulation has gathered its full speed. The table also shows the surprising fact that the intensity of the cyanide sensitive respiration attains

Table 1. *Time course of salt absorption and cyanide sensitive respiration (AR) at 18–20°C. Intact roots washed 48 hours in distilled water before the experiment. Values of Cl, K, and O₂ calculated in μmol per 1 g. fresh weight. Velocity (vel) of the processes calculated in μmol per 1 h. and 1 g. Roots rinsed 5 sec. in test solutions before start. Q Cl/AR calculated from total salt absorption up to noted interval.*

Uptake	1	5	15	30	60 minutes
Medium 0.005 M KCl					
Cl	—	2.55	4.62	6.22	8.70
K	—	2.60	3.80	5.10	6.00
AR	—	0.71	1.40	2.19	2.90
Q Cl/AR	—	3.6	3.3	2.8	3.0
Cl _{vel}	—	30.5	18.5	12.4	8.7
AR _{vel}	—	8.5	3.9	4.4	2.9
Medium 0.010 M KCl					
Cl	6.2	8.3	10.0	12.9	16.4
K	6.9	7.1	10.2	11.2	13.6
AR	—	0.76	1.45	2.8	3.85
Q Cl/AR	—	10.9	6.9	4.6	5.2
Cl _{vel}	372.0	99.5	40.0	25.8	16.4
AR _{vel}	—	9.1	5.8	4.0	3.0
Absorption and respiration in intervals. 0.010 M KCl					
Uptake	15–30 minutes		30–60 minutes		
Cl	2.9		3.5		
AR	0.53		1.05		
Q Cl/AR	5.5		3.3		

about three times higher speed 5 minutes after start as compared with the velocity of the anion respiration 60 minutes after start.

With increasing time the cyanide sensitive respiration decreases, but to a considerably lower degree than the salt absorption. The quotient $\frac{\text{absorbed anions}}{\text{consumed O}_2}$ (Q Cl/AR) is declining more rapidly in 0.005 M than in 0.010 M KCl. With 0.010 M KCl Q Cl/AR attains start values (5 minutes) of 10.9 and values exceeding 4, *viz.* “the critical value” (cf. Lundegårdh 1949 c, 1954, 1955, 1958), still result from a calculation of the *total quantity of anions absorbed in one hour*. If, however, the calculation is based on *the increments between the measured intervals* (see table 1) Q Cl/AR declines from 5.5 in the interval 15–30 minutes to only 3.3 in the interval 30–60 minutes.

The results quoted in table 1 indicate the existence of an initial period of salt absorption. This period starts immediately after the transference of the roots from distilled water to a salt solution. The extremely rapid absorption during the first minutes points to adsorption or ion exchange as the main

Table 2. *Constantly proceeding chloride accumulation in whole plants.* The initial salt absorption has been subtracted from the total absorption in 2 hours. The solution was than changed for the next interval. Here no subtraction. Similar results were obtained in experiments with cut roots. 20°C. Medium 0.005 M KCl.

	2	4 hours
Cl abs	3.54	3.55 $\mu\text{mol/h. and g.}$

process. The initial absorption soon declines and appears to be approximately finished in 15 minutes. This can be concluded from the fact that subtraction of the salt quantities absorbed during the first fifteen minutes from the total quantity taken up in 60 minutes renders values which remain constant during the following 3 to 4 hours (see below). The active accumulation starts much slower and appears to have gathered full speed in about 30 minutes from the start. This result is in agreement with previous determinations of the activation of real accumulation in very dilute solutions, in which the absorption in the initial period remains low (see Lundegårdh 1949 a). The interval 15—30 minutes comprises the transgression of declining initial absorption and accelerated accumulation.

Our distinction between an initial absorption, finished in about 15 minutes, and an active accumulation, gathering full speed in about 30 minutes, has been tested in a large series of experiments. I restrict myself here to show, in table 2, the mentioned constant proceeding of the active accumulation. In the experiments quoted in table 2 the solutions were changed in periods of 2 hours. The start period thus affects only the period 0—2 hours, not the period 2—4 hours. Subtraction of the initial absorption in the first 15 minutes from the total salt absorption in the period 0—2 hours gives the same value of accumulation as in the period 2—4 hours from which no initial absorption has to be withdrawn.

As shown in Figure 1 the complete course of salt absorption, starting from desalted roots, follows a curve, the steeply rising initial part of which corresponds to the initial absorption. The extrapolation from the approximately linear course of active accumulation (see the dotted lines in Figure 1) results in values of the ordinate which approximately coincide with the initial absorption in 15 minutes. It must then be concluded that the active accumulation shows a start lag of about fifteen minutes and that the induction period of the active accumulation is concentrated mainly to the interval 15—30 minutes. These conclusions are in agreement with earlier results as to the time of penetration of dissolved substances into the root tissue (*e.g.* Lundegårdh 1949 b).

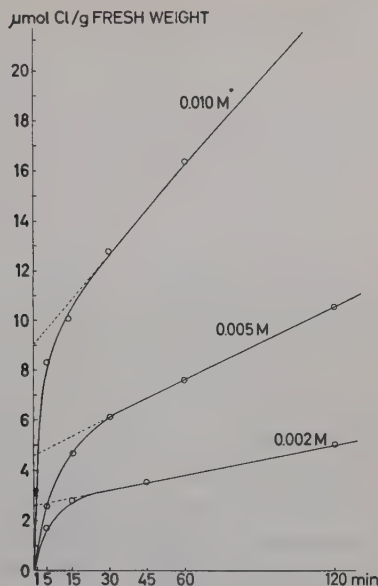


Figure 1. Time course of the absorption of chloride anions by wheat roots at different concentration of KCl in the medium. Temperature 18°C.

4. Diffusion and Adsorption

If it is hypothetically assumed that the quantities of chloride absorbed during the initial 15 minutes are uniformly distributed in the root tissue this would mean that about 60—100 per cent of the space is invaded by the external solution. In the first five minutes the corresponding figures would amount to 40—80 per cent.

It must be postulated that diffusion always participates in the movement of salts through a tissue. The following calculations may throw some light upon the capacity of diffusion.

In my experiments on the surface potential of living roots (Lundegårdh 1941) it was shown that the surface membrane of the protoplasm of the epidermis cells is the site of exchange processes between cations, *e.g.* H^+ , adsorbed to this layer and cations circulating in the external medium. The exchange processes could be followed by measuring the Donnan potential. By means of a rapidly recording cathode ray oscillograph the time elapsing between the application of a salt to the root surface and the change in potential could be accurately measured.

These experiments showed that an inorganic cation, *e.g.* Li^+ or Ca^{2+} , penetrates the cell wall in a half-time of about 1.5 to 2.0 sec. The thickness of the wall is varying but may be said approximately to amount to 0.001 mm. As to the time of entrance of the ions the results were in accord with the formula given by Perrin (see Lundegårdh 1941, p. 557) for calculation of

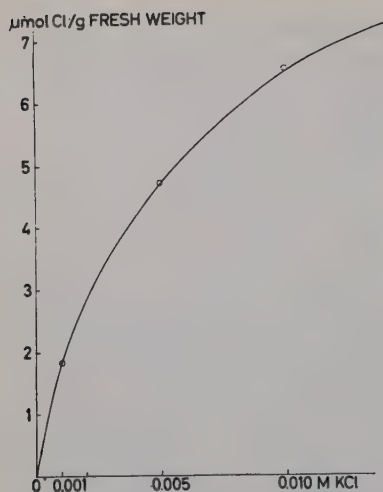


Figure 2. *The relation between chloride absorption and external concentration of KCl during the initial salt absorption of wheat roots (period 0—15 minutes). No significant difference was observed between experiments at 20° and 1—3°C.*

the average distance one particle moves through a medium of known viscosity. The distance is, according to Perrin's formula, proportional to the square root of time. Wheat roots have a radius of 0.25 mm. Provided that the average viscosity is the same as for water the calculation shows that an inorganic ion may be able to penetrate about 55 per cent of the root tissue in 15 minutes. This figure is lower than the values of initial absorption given above, but not very far from it. It must be taken into consideration, however, that the viscosity of the root cytoplasm is certainly higher than that of water.

According to our calculation diffusion would consequently even under favorable conditions, *e.g.* free mobility of the ions, be lower than half of the absorption. The actual circumstances are, however, certainly still more complicated, because it cannot be postulated that the salts move freely through the whole cell volume. The tonoplast is known to be only slowly permeable to neutral salts, *viz.* ions, and probably creates a retarding barrier against diffusion. The total volume of the cytoplasm, into which salts probably diffuse comparatively easily, amounts to only ca. 5 per cent of the root. Only a small fraction of the cells in a 60 mm. long root contain more protoplasm. As too the metabolism of different zones see Lundegårdh (1950 a).

A confinement of the initially absorbed salts to the cytoplasm would correspond to a 12-fold accumulation and a concentration of ion carriers amounting to at least 0.05—0.1 *M*. In view of the probably low content of water in the cytoplasm and the wealth of polyvalent large ions such a figure is not altogether improbable. If the initial absorption is confined mainly to the cytoplasm this would mean a considerable narrowing of the paths of diffusion and our calculation would yield still lower values. The surprising result that a linkage obviously exists between the initial absorption and the

Table 3. *Salt absorption in the presence of cyanide.* 23.5°C. Medium 0.002 *M* KCl+0.002 *M* KCN. Values in μmol , per 1 g. fr. wt.

Ion absorbed	1	5	15	30	60 minutes
Cl	0	0.42	1.50	2.30	2.53
K	0	0.43	1.50	1.90	2.30

cyanide sensitive fraction of the respiration (see Table 1) points to the co-operation of metabolically enhanced diffusion. In order to find out the magnitude of this metabolic side of the initial salt absorption experiments were performed with salt absorption in the presence of cyanide.

Previous experiments performed in this laboratory (Lundegårdh and Burström 1935) showed a certain absorption of nitrate anions in the presence of 0.001 *M*—0.006 *M* cyanide (5—16 per cent of the normal absorption). A large number of experiments, on the other hand, resulted in almost negligible quantities of chloride absorbed in the presence of cyanide. New experiments (see table 3) showed, however, that certain quantities may be absorbed in the presence of cyanide.

In Table 3 the absorption of chloride amounts to 1.5 μmol Cl per 1 g. root substance in 15 minutes. This is 50 per cent of the initial absorption in 0.002 *M* KCl. Other experiments resulted, however, in still lower values, 0.07 to 0.16 μmol , *viz.* from 2.5 to about 6 per cent. These differences are difficult to understand unless we assume that cyanide affects the basic structure of the cytoplasm more or less intensively according to the mutual concentrations of salt and cyanide, and to other more individual circumstances. The experiments show the subordinate role of pure diffusion in the initial absorption of salts. The figure 1.5 μmol per 1 g. in 15 minutes corresponds to a diffusion constant of in maximum only $0.04 \cdot 10^{-6} \text{ cm}^2 \text{ sec}^{-1}$. For comparison it may be mentioned that the diffusion constant of CO_2 in water amounts to $0.2 \cdot 10^{-5} \text{ cm}^2 \cdot \text{sec}^{-1}$, or about five times more than our maximum figure. It must hence be concluded that powerful barriers to free diffusion exist in the root tissue, which has been treated with low concentrations of potassium cyanide.

Even if it will seem somewhat hazardous to generalize these results it may be inferred from the experiments in this paragraph that both diffusion and "adsorption" participates in the initial absorption and that diffusion is probably the least important pathway of the distribution of salts in the protoplasm.

5. Respiratory Sensitivity of the Initial Absorption

The observed *burst of respiratory activity in the first minutes of initial salt absorption* (Table 1) may be explained as a salt activation of the cytochrome

system. This phenomenon has been studied in wheat homogenates (Lundegårdh 1953, Butler 1953) and in pure preparations of cytochrome c (Boeri & Tosi 1954). I have explained the activation out from an electrochemical interpretation of the electron transference, *viz.* a shuffling of anions in the surroundings against electrons moving in the enzyme system (see Lundegårdh 1954, 1955, 1958). Because large quantities of salt anions are simultaneously adsorbed to cytoplasmic carriers, but no real accumulation occurs in the initial period, no conclusions can be drawn from the relation $\frac{\text{absorbed anions}}{\text{consumed oxygen}}$ prevailing in this period of salt absorption. As mentioned in the preceding discussion the mere fact that most of the initial absorption stops in the presence of cyanide points to a remarkable sensitivity of the power of initial salt absorption to CN.

Because inhibition occurs at concentrations of cyanide below those of KCl the phenomenon cannot be simply a competitive one. Blocking of the active iron of the hemin enzymes may be responsible only for a negligible part of the total inhibition, because the concentration of cytochrome+peroxidase only amounts to about 0.2 to 0.3 μmol per 1 g. root substance. A tentative explanation of the large drop in initial absorption following a treatment with cyanide is inactivation of ion carriers simultaneously with inactivation of the cytochrome system+peroxidase.

The carriers are probably more or less identical with the organized body of the cytoplasm. It can be inferred from scattered observations in the literature that the delicate structure of the protoplasm is sensitive to disturbances in the high energy phosphate metabolism. It is known from biochemical investigations that the activity of the intact cytochrome system is one of the main sources of high energy phosphorylation. Even inhibitors which inactivate only special links in the respiratory system, *e.g.* dinitrophenol and fluoride (Lundegårdh 1949 b, 1952 etc.), severely affect the salt absorption. Dinitrophenol is also known to retard the transport of salts in the cytoplasm (Arisz 1953, Butler 1953), facts which are in good agreement with our conclusion that undisturbed respiration is a necessary condition for the salt carrying functions of the cytoplasm. This conclusion in particular refers to anion carriers. Cations are to a certain extent absorbed also at inhibited respiration.

6. Chloride Absorption and Anion Respiration at Low Temperature

The experiments quoted in table 1 were repeated at temperatures near zero degree C. One of these experiments is shown in table 4. Q_{10} of the anion respiration amounts to the "normal" value 2.20 to 2.45. An important result of the

Table 4. *Time course of salt absorption and cyanide sensitive respiration (AR) at +1°C. Values in μmol per 1 g. fr. wt. Velocity calculated in μmol per g. and h. Medium 0.005 M KCl.*

Uptake	5	15	30	60 minutes
Cl.....	3.7	5.3	6.9	8.2
K.....	5.1	4.6	7.0	8.4
AR.....	0.19	0.40	0.62	1.04
Cl _{vel}	46.7	21.2	13.8	8.2
AR _{vel}	2.3	1.6	1.2	1.0
Q Cl/AR (total absorption).....	20.5	13.2	10.9	7.9
				30—60 minutes
Q Cl/AR (period of accumulation).....				3.1

low temperature experiments is the fact that the initial salt absorption is very little affected (*cf.* 2). Table 5 shows that the active accumulation, calculated from the period 15—120 minutes, is comparatively little affected by changes in the temperature, too, as long as the KCl concentration in the medium remains low. At 0.010 M KCl Q_{10} of Cl absorption amounts to 1.7. Table 5 also shows that Q Cl/AR rises from 0.78—1.88 to 2.72—3.20 at a drop of the temperature from 21° to 3° above zero C. The “critical” value 4 was thus not surpassed. If Q an/AR had been calculated from the *total* absorption in 0—120 minutes, as made by Sutcliffe and Hackett (1957) and some zoological biochemists, values near 8 would result, but these values are not significant for the process of accumulation.

The experiments quoted in table 5 show a strong effect of the salt concentration in the medium on the velocity of active accumulation. Fig. 2 gives corresponding values for the initial absorption. Table 5 teaches us that at 21° the velocity of accumulation is controlled by the velocity of initial

Table 5. *Influence of the temperature on the period of salt accumulation. Values calculated for the period 15—120 min. (initial period omitted). Values in μmol per h. and g. Cyanide sensitive respiration=AR.*

Medium KCl M	+ 3°C			+ 21°C		
	Cl abs.	AR	Q Cl/AR	Cl abs.	AR	Q Cl/AR
0.002	1.83	0.65	2.72	1.80	2.32	0.78
0.005	2.62	0.80	3.27	3.12	2.52	1.24
0.010	2.87	0.90	3.20	6.27	3.33	1.88
	Q_{10} Cl			Q_{10} AR		
0.002	1.0			2.43		
0.005	1.1			2.20		
0.010	1.7			2.45		

absorption. Other experiments show a nearly linear relation between external concentration and velocity of accumulation up to about 0.005 *M* KCl (see also Lundegårdh 1949 a). At +3°C., however, the accumulation cannot keep pace with the initial absorption, obviously because the velocity of the anion respiration is too low at this temperature. This is an example of the cytochrome system as pacemaker of salt accumulation.

7. Osmotic Work Performed During Active Accumulation

If the salt absorption is calculated per volume tissue and referred to the concentration of the medium the period of *initial absorption* (0—15 minutes) never leads to real accumulation, *viz.* values exceeding 100 per cent (see above). It was concluded that the initial absorption is most probably confined to the cytoplasm only and does not penetrate the tonoplast barrier. Calculated per volume cytoplasm the initial absorption certainly rises to concentrations which are considerably higher than those of the medium (see above), a phenomenon which we have ascribed to the high carrier capacity.

For *real accumulation* in the large sap spaces of the root cells the mechanism of active accumulation must be at work. It resulted in a total accumulation of 280—420 per cent (Table 6) and a rise of the osmotic pressure of 1.2 to 1.5 atmospheres. The pressure here probably sets a limit to the accumulation. It is observed that the end pressures are not very different in 0.005 and 0.010 *M* KCl.

8. Concluding Remarks

Earlier work in this laboratory showed the power of adsorption and exchange of cations. The new experiments show the same also for chloride anions. The dual carrier capacity of the cytoplasm is reminiscent of charcoal. The dominance of cation carriers in the surface layer of the root cytoplasm does obviously not, as believed earlier (Lundegårdh 1940), retard the entrance of anions. My observations (Lundegårdh 1950 a) as to the free mobility of both anions and cations inside of the root epidermis pointed, however, to a similar result, *viz.* the power of the internal cytoplasm to carry and transport both anions and cations. Other anion carriers than the hemin enzymes are not exactly known, but most constituents of the protoplasm, including proteins, behave as "Zwitterions". The fact that absorbed chloride only with difficulty can be removed from wheat root tissue points to the existence of specific anion carriers and to a partly strong linkage between anion and carrier.

Table 6. *Total absorption of chloride in a period of 4—5 hours. Values in μmol per 1 g. fr. wt.*

Medium (KCl)	0.005 M	0.010 M
Factor of active accumulation $\left(\frac{\text{internal Cl}}{\text{external Cl}}\right)$ in %	420	280
Osmotic value of accumulated salt	1.2 atm.	1.5 atm.

The vindicators of the conception "free space" (see Briggs and Robertson 1957) seem to believe in a more or less free flow of salts from the medium into the cytoplasm. The barrier formed by the cytoplasmic membranes of the cells, which are in direct contact with the medium, *e.g.* the epidermis of the roots, can, however, not be ignored. The fact that once absorbed salts are only slowly returned to the medium illustrates a special barrier function which distinguishes this boundary from the boundaries between the cells of the cortex.

The statement of a comparatively strong initial absorption of salts, together with my earlier discovery of the pronounced nonmetabolic mobility of neutral salts in the root cortex (Lundegårdh 1950 a; *cf.* also 1945) opens new aspects as to the possibility of the ascending sap stream to transfer salts from the medium to the aerial parts independently of the anion respiration. Hylmö (1953), Kylin and Hylmö (1957) and others attribute a comparatively prominent role to such a side-way salt stream through the 'free space'. More experimental work is, however, needed to find out the real capacity of this stream.

The conception "non-metabolic transport" is, according to the results of the present investigation, not quite adequate. My results from 1950 positively show the existence of a "non-metabolic" transport of salts in the cortex. But a continuous salt stream demands *continuous initial reabsorption from the medium*. And we have now seen that this absorption is not wholly "non-metabolic", because the power of adsorption, *viz.* the carrier function of the cytoplasm, postulates the activity of the cytochrome system. It was shown that diffusion in itself probably plays a more subordinate role in the transport of salts through the root tissue. My earlier findings of a retardation of the salt flow under the influence of cyanide may not exclusively depend on inhibited accumulation but also on a narrowing of the carrier-pathways through the tissue. The existence of a "direct line of salt transport" does of course not rule out the process of active accumulation from its role as a promotor of the ascending sap stream. The active accumulation, which produces osmotic power, fills the sap spaces of the root cells and thus creates the high level of salt concentration which in its turn speeds up the flow of

salts into the central vessels and channel. I have earlier shown that this pool of accumulated salts is needed for a continued saltflow at occasional absence of salts from the medium.

One of the main results of this investigation is the statement of an all-round activity of the cytochrome system in salt transport. Two sides of the activity of the cytochrome system are here co-working, *viz.* 1. the maintenance of an appropriate level of initial salt absorption, and 2. the anion respiration. The latter is figured taking over salt ions from the carrier structure of the cytoplasm and to accumulate them in the sap space. The role of the mitochondria and of the tonoplast in this process is not accurately known. An established fact is, however, the quantitative relations between the quantity of accumulated salts and the intensity of the cyanide sensitive respiration.

According to the theory of anion respiration the structural organization of the cytochrome system promotes a polar transport of anions in electrochemical counterbalance to the transport of electrons through the system. Theoretically in maximum four anions may be transported for one molecule of oxygen consumed, *viz.* $Q \text{ an/AR}=4$. The main argument hitherto raised *against* the theory of anion respiration is the fact that this quotient, if it is calculated from the total salt absorption from start to end, sometimes exceeds the value four. Values of 6—7 have been published, *e.g.* by Sutcliffe and Hackett (1957). In the present investigation still higher values were obtained from experiments at low temperature. An inappropriate experimental technique has however misled Sutcliffe and Hackett. They are overlooking the initial absorption, a process which in itself does not carry on active accumulation. If this fact is taken into consideration the argumentation of Sutcliffe and Hackett is invalid.

Sutcliffe and Hackett (1957) present as a second argument against the theory of anion respiration the observation by Robertson, Wilkins and Weeks (1951) that dinitrophenol may inhibit salt absorption at still maintained cyanide sensitive respiration. Sutcliffe and Hackett are here ignoring my reinvestigation of the case (1952 and later). I was able to show that dinitrophenol uncouples cytochrome b from the rest of the system. Because cytochrome b obviously participates in the control of the synthesis of high energy phosphate a retardation of its activity may severely affect the structural qualities of the protoplasm. And the maintenance of the structural qualities of the protoplasm is a necessary condition for both "passive" and "active" salt transport. I am surprised of the eagerness with which even biochemists lacking own experimental experience in the field of salt accumulation, (*e.g.* Chance and Williams 1956) have adopted the hypothesis that high energy phosphorylation in itself could serve as a mechanism of salt accumulation. No scheme has been presented how such a mechanism would work. Chance (1956)

tries to minimise the importance of electron transport as motive power of salt accumulation. He claims that an internal transport of electrons through a chain of enzyme molecules does not affect the electrochemical balance of ions in the surroundings. Chance is here overlooking the fact that the start points and the end-points of the stream of electrons are in open contact with the surroundings, *viz.* the succinic acid on one end and oxygen on the other end. An internal flow of electrons through the system of respiratory enzymes will thus inevitably convey a reversed stream of anions in the surroundings. This is an electrochemical axiom.

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Studies on the Formation of Protochlorophyll and Chlorophyll *a* under Varying Light Treatments

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In previous papers by Withrow *et al.* (42) and Virgin (38) preliminary experiments were reported, showing that in the process of greening (chlorophyll *a* formation) a mechanism is involved which is governed by a low-energy requiring light reaction. This reaction is probably of the same nature as that which has been found to play an important rôle in a great many photomorphogenic phenomena (for references, see Withrow and Price, 41). The present paper is a closer study of the conditions for pigment formation in irradiated, previously dark-grown wheat seedlings with special reference to this reaction.

Material and Methods

Material. The experimental material consisted of dark-grown seedlings of wheat (Weibull's orig. "Eroica II"). The seeds were soaked for 24 hours in tap water, thereafter placed on a wire screen of stainless steel mounted on glass legs, four centimeters long. This device was placed in a round glass vessel with a lid, leaving about one centimeter between the lid and the seed bed. The diameter of the vessel was 11 centimeters. Nutrient solution according to Kopp (24) was added up to the wire screen. The seeds were left to germinate in darkness in an incubator placed in a thermostatically controlled darkroom (21°C). After another 24 hours the lid was taken off the vessel and the seedlings were left for seven days in darkness. No extra watering was required during this period. The plants suitable for experiments had then reached an average total length of 21 centimeters. The average length of the coleoptiles amounted to 8 centimeters. For experimental purposes leaves were excised from the plant about two centimeters above the apex of the coleoptile and about

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two centimeters of their tips were cut off. The pieces of leaves thus obtained were about 9 centimeters in length and quite homogeneous. During the handling of material in darkness very dim green light was used, obtained from a flashlight provided with an interference filter with a maximum transmission at 500 m μ . As the half value width for the transmission does not exceed 20 m μ , the transmitted light should be within the range without any strong physiological effect (Withrow and Price, 41). Checks on pigment formation gave no indication of any effect.

During irradiation the leaves were spread out in a single layer on moist filter paper at the bottom of a glass trough covered with a glass plate. All irradiation experiments were performed at a temperature of 21–22°C.

Light source and filters. The main light source was a 1000-watt projection lamp (Philips, type 294 G). Although the emitted energy from an incandescent lamp increases towards longer wave lengths, appropriate filters could be found to absorb or reflect all of the infrared irradiation, as desired. All light was filtered through a layer of running water, ten centimeters in thickness. The pigment formation was studied in blue and in red light free from infrared. The filters used for red light were in some instances an interference filter (662 m μ) combined with copper sulphate solution plus a heat absorbing interference filter (G.A.B. Reflexionswärmefilter. "Cal-flex"). On other occasions a filter OR 1 (Chance Brothers Ltd) was used instead of the red interference filter. When blue light was used, this was obtained by using a copper sulphate solution combined with a solution of "patent blue". The transmission curves for the different filter combinations can be seen in Figure 1. The proper filter combinations used in the various experiments reported in the following will be mentioned in the Figures in connection with the description of the single experiments.

The light from the lamp was focused upon the plant material by means of a lens system and a mirror. The intensity of the light was measured by means of a Moll thermopile combined with a mirror galvanometer.

Pigment determination. The pigment concentration was determined spectrophotometrically on ether extracts of the irradiated leaves. About 400 milligrams of leaf tissue were ground with sand in a glass mortar together with ten milliliters of acetone. The slurry thus obtained was filtered through a glass filter. The residue and the mortar were washed three times with acetone until 20 milliliters of acetone were used. Fifteen milliliters of ether were then added to the filtrate. The ether-acetone extract was then washed three times with distilled water in a separatory funnel and finally run into a graduated stoppered cylinder. The end volume of the ether extract was kept between 10 and 15 milliliters. Immediately after the extraction the ether extract was placed in a dark cold room at +4°C. Eventual turbidity due to water droplets disappeared some hours after this treatment. In most cases the irradiated plant material was placed for a few days in a deep-freezer at -30°C prior to extraction. This period in the cold did not measurably change the initial pigment concentration. In the experimental series where protochlorophyll was determined, the grinding of the material with acetone took place in very dim green light.

Chlorophyll *a* was determined at the wave length 662 m μ and protochlorophyll at 623 m μ . In the calculation of the absolute amounts the values 95 and 36.2, respectively, were used as the specific absorption coefficients for the two pigments. In the calculation of the protochlorophyll concentration the value was corrected for the simultaneously present chlorophyll *a*, using the value 29.9 as the specific absorption

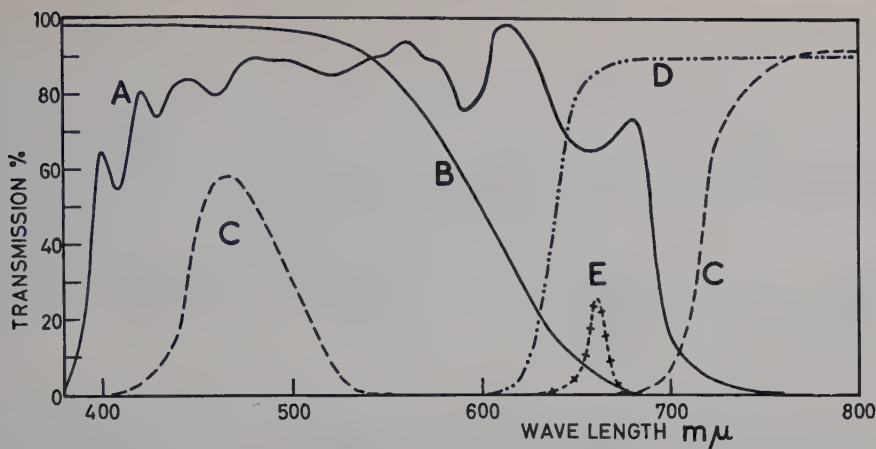


Figure 1. Transmission of the different filters used in the present investigation. A: Heat reflexion filter "Calflex". B: $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$, 25 g. per liter. C: Patent blue, 200 mg. per liter. D: OR 1 (Chance Bros.). E: Interference filter. The values were obtained with a Beckman spectrophotometer, Model DU, (1-cm. path). The curves show the percentage transmission of the filters for different wave lengths. When using a light source with increasing output of energy towards the red (an incandescent lamp in the present study) the absolute transmission towards the red part of the spectrum will become proportionally greater than indicated by transmission curves on percentage basis. This is taken into consideration when evaluating the light transmitted by the different filter combinations used.

coefficient for this pigment at the wave length 623 mμ. The method used for calculation was principally the same as described by Koski *et al.* 26.

Chlorophyll *b* — when present — occurred in such small amounts that it did not interfere with the measurements.

Experimental

a. Protochlorophyll formation during the process of greening

The formation of chlorophyll *a* in a previously dark-grown plant during irradiation with white light is a complicated process. After the rapid transformation of the protochlorophyll already present in dark, (Koski *et al.*, 26; Smith and Benitez, 33; Virgin, 36, 37; Shibata, 32) the further formation of chlorophyll *a*, *i.e.* the process of greening, is much slower and can be divided into two markedly different steps. During the first two to three hours in light the rate of formation is very slow (Blaauw-Jansen *et al.*, 2; Virgin, 37), whereafter an acceleration of the formation sets in. It has earlier been shown (Virgin, 37) that the rate of the formation of chlorophyll *a* during the first two to three hours is approximately the same as the rate of protochlorophyll formation in darkness. Hence the chlorophyll *a* during this first period of the

Table 1. *The influence of different light and dark treatments on the formation of protochlorophyll in previously dark-grown leaves of wheat. (Four series of each treatment.)*

Intensity of red irradiation 320 ergs/cm.² · sec.

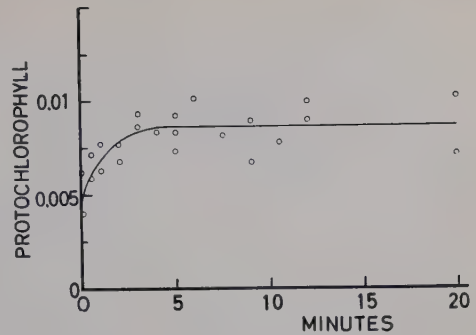
Red light (3 min.) + 100 f. c. white light (15 min.)	Red light (3 min.) + darkness (6 hours) + 100 f. c. white light (15 min.)	Red light (3 min.) + 100 f. c. white light (15 min.) + darkness (1 hour)	Red light (3 min.) + darkness (6 hours) + 100 f. c. white light (15 min.) + darkness (1 hour)
0.0010 mg./g. leaf	0.0010 mg./g. leaf	0.0070 mg./g. leaf	0.0099 mg./g. leaf
0.0016 "	0.0009 "	0.0072 "	0.0101 "
0.0013 "	0.0010 "	0.0094 "	0.0114 "
0.0017 "	0.0020 "	0.0065 "	0.0130 "

greening is with all probability formed via protochlorophyll. Whether this is the case also in the later stages of the greening could not be proved with the method earlier used. The reason for this was the impossibility of determining small amounts of protochlorophyll in the presence of large amounts of chlorophyll *a*.

The length of the lag phase in chlorophyll formation during the beginning of irradiation has been shown to be affected by a short pre-irradiation (Virgin, 38), wherein red light is the most effective (Withrow *et al.*, 42). A short impulse of red light followed by five to six hours of darkness thus causes an immediate rapid formation of chlorophyll *a* during a subsequent period of continuous irradiation. Under these conditions the lag phase in chlorophyll synthesis completely disappears and the rate of chlorophyll formation is high from the onset of the irradiation. This fact enabled the determination of the formation of protochlorophyll also during the later stages of the greening, as will be shown below.

The protochlorophyll present in a dark-grown plant disappears almost completely after an irradiation for only a few minutes with medium intensities of light (Koski, 25; Smith and Benitez, 33; Virgin, 36, 37; Shibata, 32). If the plant is afterwards replaced in darkness, the formation of protochlorophyll takes place again, first rather rapidly, then more slowly until the initial concentration level is reached (Virgin, 37). This process takes about eight hours at room temperature (20°C). If the formation of chlorophyll *a* in light always goes via protochlorophyll, the rate of protochlorophyll formation after a red light treatment should be more rapid than without. That such is the case is shown in Table 1., where also the different treatments given to the leaves can be seen. The first two columns from the left are controls showing that only small amounts of protochlorophyll are left in the leaves immediately after an irradiation for 15 minutes with white incandescent light with an illuminance of 100 foot candles. Most of this protochlorophyll is probably the

Figure 2. *The effect of the length of the irradiation period on protochlorophyll formation.* Dark-grown leaves of wheat were irradiated with red light. Filters: A+D+E (cf. Figure 1). Light intensity 320 ergs/cm.² · sec. The time of irradiation is denoted along the abscissa. After the irradiation the leaves were kept in darkness for five hours and then irradiated for 15 minutes with white incandescent light (100 f.c.). After another hour in darkness the content of protochlorophyll was determined. (In the Figure denoted as mg. per g. of fresh weight.)



part of the two holochromes not transformable by light (Shibata, 32). The pretreatment prior to the irradiation is thus unimportant in this respect. The transformation of protochlorophyll to chlorophyll *a* has taken place to about 90 per cent which is in good agreement with the data given by Smith and Benitez (33) for leaves of etiolated barley. When the leaves had been allowed to remain in darkness for one hour after the last light treatment (column three from the left), protochlorophyll was resynthesized to some extent (cf. Virgin, 37). If the same treatments were given except for an addition of a dark period for six hours between the first red light impulse and the irradiation with white light, the rate of the resynthesis of protochlorophyll during the subsequent dark period was increased considerably. *This implies that the effect of red light on chlorophyll formation is due to its action on protochlorophyll or some precursor to protochlorophyll.*

Another conclusion which may be drawn from these experiments is that *chlorophyll a in a plant in light during the process of greening is always formed via protochlorophyll.* It is always the rate of the formation of this substance which determines the rate of chlorophyll *a* formation.

The effect of the short red light impulse on protochlorophyll formation has reached its maximum value after a rather short period of irradiation, as shown in Figure 2. The intensity of the irradiation was in this case 320 ergs/cm.² sec. The light was obtained by means of an interference filter with the maximum transmission at 662 mμ. In this particular experiment maximal effect was reached after an irradiation for three to four minutes. The general course of the reaction is strikingly similar to the result obtained by Borthwick *et al.* (7) in respect to seed germination. In later experiments (see below) where only the formation of chlorophyll *a* was studied (formed via protochlorophyll), higher light intensities were administered and consequently shorter periods of irradiation were required to reach a maximal response. This is probably attained when all the red-absorbing pigment in the tissue is transformed into far-red absorbing form (cf. Downs *et al.* 10).

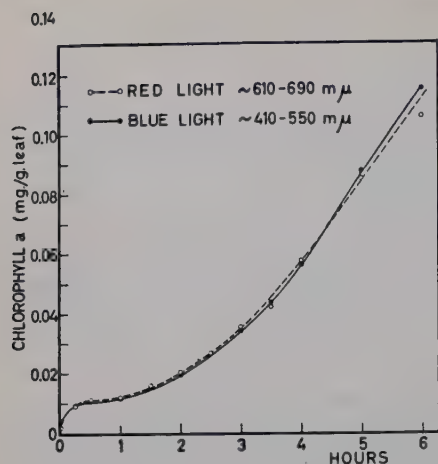
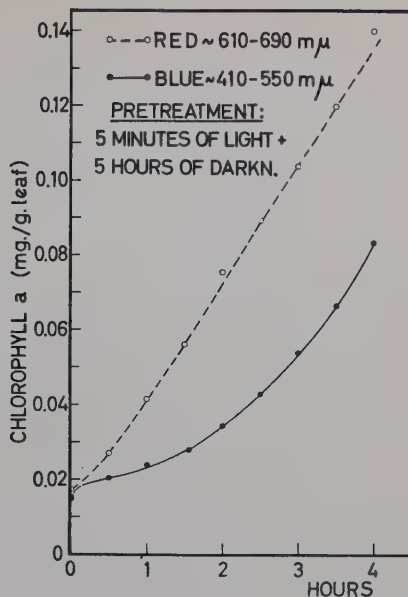


Figure 3. The formation of chlorophyll *a* in previously dark-grown leaves of wheat during continuous irradiation with blue and red light. The filters for blue light consisted of A+B (2-cm.) + C (2-cm.) (cf. Fig. 1). Light intensity 410 ergs/cm.² · sec. The filters for red light consisted of A+B (2-cm.) + D (cf. Figure 1). Light intensity 420 ergs/cm.² · sec.

b. Greening in different parts of the spectrum

From works by Frank (14) and Koski *et al.* (26) it is evident that the action spectrum for the formation of chlorophyll *a* is practically identical with the absorption spectrum for protochlorophyll. This implies that the light energy used for the transformation is absorbed by the protochlorophyll and that no other pigment system is involved in this transformation. It was shown above in the present paper that the chlorophyll *a* formed during the process of greening originates from protochlorophyll. Any change in the rate of the process of greening can therefore be referred to changes in the rate of formation of protochlorophyll. Since the formation of chlorophyll *a* shows a considerable acceleration after a few hours of continuous irradiation with white light and, as shown above, a pretreatment with red light strongly effects the protochlorophyll formation, one might expect that this acceleration is caused by the red light present in the white light used for the irradiation experiments. If this assumption is correct, one should get lower greening rates in blue light than when red light is employed. Irradiation with only blue light should give no acceleration in chlorophyll formation after two to three hours of continuous irradiation. The results from such a series of experiments are shown in Figure 3. Dark-grown leaves have been continuously irradiated with red light free from blue and infrared light and with blue light free from red and infrared light. The wave length ranges used are rather wide and cover more or less completely both of the two main absorption bands of the protochlorophyll (cf. Koski *et al.*, 26). The energy content of the red and blue light was adjusted to give the same amount of chlorophyll *a* after an irradiation for two hours with the two types of light, respectively. By mere chance the energy happened to be the same in the two cases. From the Figure it is clear that one

Figure 4. The formation of chlorophyll *a* in dark-grown leaves of wheat during continuous irradiation. Leaves were given a short impulse of red and blue light, respectively, five hours prior to continuous irradiation. Filters and light intensity as in Figure 3.



gets exactly the same rate of chlorophyll *a* formation in the two cases, *i.e.*, an acceleration in the formation sets in also, when only blue light is used. This implies that the given assumption must be wrong, that much stray light is present in the blue light, or that blue light also has an effect on the rate of chlorophyll formation. As thermopile measurements with different filters gave no trace of irradiation beyond the given wave length limits, the results seemed rather puzzling. In order to check the effect of the light used on the lag phase, another set of experiments were arranged. The results of these can be seen in Figure 4. From the experimental results reported in this diagram the following conclusions may be drawn. A short impulse of red light followed by a dark period gives a very high rate of chlorophyll *a* formation during a subsequent period of continuous irradiation with the same light. This is in full accordance with what we know about the effect of a short red light impulse (Withrow *et al.*, 42). If the same treatments are given with blue light, however, one can not detect any effect of the blue light impulse. This also shows that the action spectrum for producing an acceleration in chlorophyll formation (properly speaking: protochlorophyll formation) has its greatest peak in red (*cf.* the action spectrum for photomorphogenic changes given by Withrow *et al.*, 41). The only difference between the curves presented in Figure 3 and Figure 4 is that the initial amount of chlorophyll in the latter instance is higher. This depends on the fact that some of the protochlorophyll present in the dark was transformed during the first short light

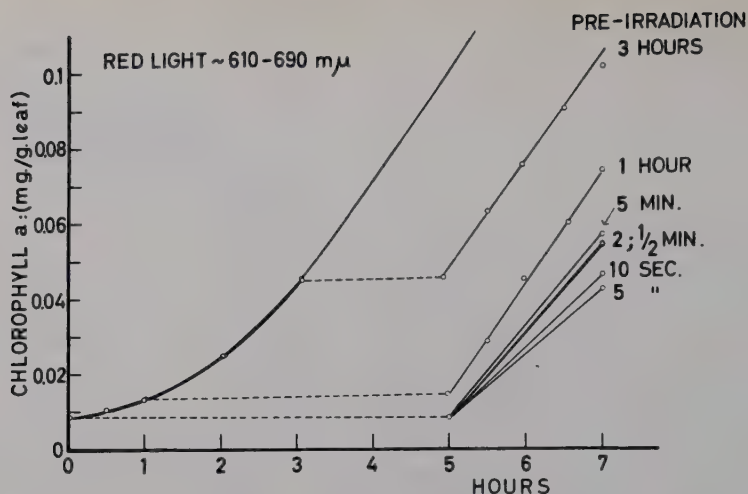


Figure 5. The formation of chlorophyll *a* in red light under varying light and dark treatments. Dark-grown leaves of wheat were irradiated for varying lengths of time followed by dark periods. Thereafter constant light was given for two consecutive hours. Filters and light intensity as in Figure 3.

impulse and that additional protochlorophyll has been formed during the following long dark period. This additional protochlorophyll has in turn been transformed into chlorophyll *a* during the first few minutes of the subsequent continuous irradiation. Disregarding this difference, however, the behaviour in blue light in the two cases is exactly the same. The fact thus remains that it is only the lag phase in chlorophyll formation which is affected by red light. After the end of this period the rate of pigment formation is the same in blue and red light, *i.e.*, an acceleration in the chlorophyll formation in both cases. The question is thus still unanswered as to why the acceleration sets in when blue light is used. The results obtained do not exclude, however, an effect of blue light. Let us assume that blue light has an effect but that this is of a very short duration. Then a short impulse of this light would have no effect after a subsequent long period in darkness; whereas the effect of a similar impulse of red light would persist and build up a reaction during the dark period, as has been shown to be the case. A *continuous* irradiation with blue light, however, would give an acceleration in the pigment formation, because in this case the reaction products might increase and gradually reach the level which is the prerequisite for an acceleration in the rate of pigment formation. A way of finding out the facts in this respect is to irradiate the material for some hours with blue and red light respectively. After allowing the material to remain in darkness for some time the irradiation should be continued. In this way it would be possible to determine the duration of the

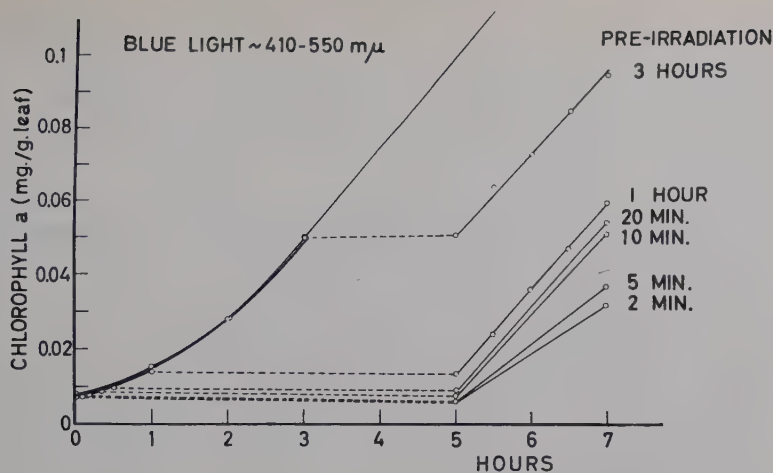


Figure 6. The formation of chlorophyll *a* in blue light under varying light and dark treatments. Otherwise as in Figure 5.

effect of red and blue light. The results from a series of such experiments are presented in Figures 5 and 6. In these experiments leaves have been irradiated for various periods of time with red and blue light, respectively. The intensities of the red and blue light were adjusted so that the rate of chlorophyll *a* formation was the same during an irradiation for four hours with the two types of light (*cf.* Figure 3). At the end of the irradiation period the leaves were placed in darkness and thereafter irradiated for two hours with the same light as used for the first irradiation. The second irradiation always began five hours after the start of the first light impulse. Except for a few experiments, chlorophyll determinations were performed only after the end of the two-hour irradiation period. Control measurements (the curves for one and three hours of pre-irradiation) showed that the amount of chlorophyll at the start of the second irradiation period was always the same as the amount obtained after the first irradiation. This implies that no formation or decomposition of chlorophyll takes place during the stay in darkness. Small amounts of protochlorophyll were formed, however, during the dark period (*cf.* Virgin, 38). A striking fact shown in the Figure is that above a certain period of irradiation no further increase in the rate of chlorophyll formation is obtained; the curves representing the formation of pigment follow a linear course parallel to each other, the rate of formation being the same as that after a continuous irradiation for three to four hours. There seems thus to be a saturation value for the period of irradiation above which a further increase does not give any rise in the response. Below this value a decrease in the period of irradiation will cause a reduction in chlorophyll *a* formation.

Exactly the same pattern is obtained with red light as with blue light. The only difference is that the time required for reaching the saturation value is between 10 and 30 seconds for red light, but between 5 and 10 minutes for blue light. There is thus a difference in sensitivity for the two light qualities which is as about 22 : 1.

It was earlier shown that the maximal effect of a previously administered short period of irradiation is reached after a stay in darkness for five to six hours (Virgin, 38; Figure 1). Thereafter the effect on the formation slowly disappears. In Figures 7 and 8 experimental data are presented which further illustrate the effect on chlorophyll *a* formation of the length of the pre-irradiation together with the effect of varying lengths of the period in darkness. The experiments have been arranged in the following way. Dark-grown leaves were irradiated for a short period of time (from five seconds up to five minutes when red light was used — Figure 7 — and from 2 to 20 minutes when blue light was used — Figure 8) followed by dark periods varying from one to five hours. The leaves were thereafter subjected to continuous irradiation for two hours. The amount of chlorophyll *a* in the leaves was determined at the end of this irradiation. The intensity of the light was chosen to give the same rate of chlorophyll formation as when continuously administered to previously dark-grown leaves (*cf.* Figure 3). From these measurements it is also obvious that a prolongation of the period of pre-irradiation above a certain value does not further increase the pigment yield during a subsequent period of continuous irradiation. If the time between the first light impulse and the irradiation for two hours is increased, it is true that the yield will become greater, but also here one seems to reach a value which remains constant regardless of the amount of light energy given. Whatever occurs during the period in darkness when the reaction is "building up", it is thus evident that if enough light energy is administered to give maximal effect after five hours in darkness, the time course of the reaction is the same for all light energies above this saturation value. When the amount of light energy is decreased below this saturation value, the effect on pigment formation will also become diminished more or less proportionally to the amount of light given. This is in accordance with the idea of a pigment transformation taking place under the influence of red irradiance (See under "Discussion").

From the experiments reported here, it may be concluded regarding the effect on the lag phase in chlorophyll *a* (protophyll-) formation that the difference in the effect of blue and red light is only of quantitative nature. When medium intensities of light are given, the saturation value for the effect is already reached after such a short period of irradiation — this is also the case with blue light — that the further course of the pigment formation in continuous light will become the same in both cases. The effect of a pre-irra-

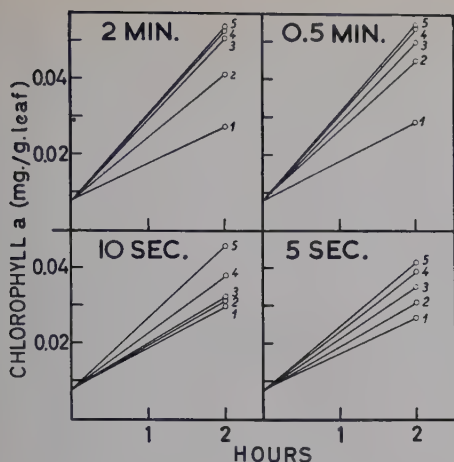


Fig. 7.

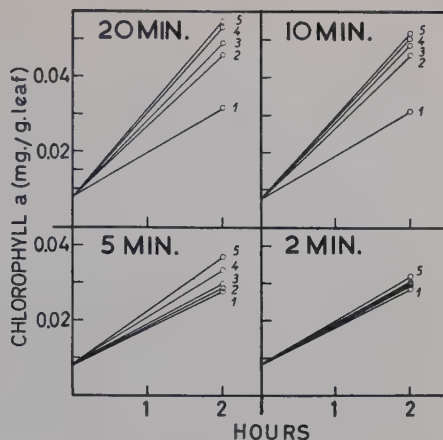


Fig. 8.

Figure 7. The formation of chlorophyll *a* during two hours of irradiation with red light. Prior to the irradiation of the leaves a short impulse of red light was given varying from five seconds to two minutes. After this light impulse the leaves were kept in darkness for one to five hours, this period being indicated in the Figure with small numerals after every single series of experiments. Filters and light intensity as in Figure 3.

Figure 8. The formation of chlorophyll *a* during two hours of irradiation with blue light. Pretreatment as in Figure 7 except that the length of the short light impulse was here varied between two and twenty minutes. Filters and light intensity as in Figure 2.

diation on the pigment formation in respect to light quality will thus become particularly obvious only when light is given for short periods of time. The consequence of this will be further dealt with in the next Chapter.

Discussion

Our knowledge of light reactions which have been found to have an action spectrum with a maximum in the red around 660 $m\mu$ is steadily increasing. Since the establishment of an action spectrum of this type for seed germination and photoperiodism (Borthwick *et al.*, 4, 6) a great many phenomena — seemingly not related to each other — have been shown to follow the same scheme. Comprehensive reviews are given by Borthwick *et al.* (7); Toole *et al.*, (34, 35), Evenari (11), and Borthwick *et al.* (5). Through studies on particularly light-dependent seed germination and internode elongation of bean seedlings (Downs, 9) and on the straightenings of the so-called "bean-hook"

(Klein *et al.*, 22, 23) it has been possible to get some idea of the mechanism. According to Borthwick *et al.* (4, 5, 6) the phenomena, particularly the reversibility of the reactions with far red, can be explained by the following assumption of pigment transformation: red light is absorbed by a red-absorbing pigment causing this to be transformed into a far-red absorbing form, the latter being the physiologically active agent in the process.

The reactions studied in the present paper have been shown to follow the given schedule (Withrow *et al.*, 42; Virgin, 38). It can therefore be considered a link in or at least to be connected with the red and far-red reaction chain. It is of particular interest that here there is also a saturation value for the amount of light energy required to obtain a maximum response. This fact strongly supports the idea of a pigment transformation.

Although red light seems to be around 20 to 30 times more effective than the same amount of blue light, looked upon from an energy basis, the effect of the latter must be taken into account when it is a question of longer periods of irradiation. The amount of light required to produce an effect on protochlorophyll formation is comparatively low. This implies that also blue light of medium intensities administered for not too short a time has an appreciable effect. The sensitivity to red and blue light differs considerably in other red-sensitive processes with the same pigment mechanism. In photoperiodism, for example, red light has been found to be 200 times as effective as blue light for the short day plant Wintex barley, while it is only about 20 times as effective for the short day plant Biloxi soybean (Borthwick *et al.*, 3; Figure 8). There is in the literature concerning photoperiodism some evidence for that blue light can give strong photoperiodic response, particularly after longer periods of irradiation (*cf.* Wassink and Stolwijk, 40). In some cases the results may be due to lack in spectral purity, but this can hardly be the case in all reported instances. Particularly the plants belonging to "group IV" according to Funke (15) show a strong photoperiodic response to blue light. It is thus evident that one must take into account the effect of blue light in the photomorphogenic response more than hitherto has been done, particularly when the period of irradiation is long. In this connection a paper by Evenari (13) should be mentioned showing a pronounced effect of blue light on seed germination. Whether these differences in the response to blue light are due to the presence of varying amounts of other screening pigments or can be referred to other factors (*cf.* Borthwick *et al.* 7, p. 221) is an open question.

In the photoperiodic response it is more difficult than in the case of effects on pigment formation to establish how long a time in darkness is required for the effect of a previous light impulse to reach its maximum value. From the

well-known fact that a light impulse given to short day plant during the dark period has the greatest effect when administered after about six hours in darkness (Harder *et al.*, 16; Carr, 8), the conclusion may be drawn (Hendricks, 19) that the reason why a plant blooms or fails to bloom under the influence of long nights depends upon the level of the materials essential for flowering, arising from the reaction controlled by the far-red absorbing pigment. Just a very short light impulse will then suddenly increase the amount of this pigment and change the subsequent reactions. It is now of interest to find that in respect to effects on chlorophyll formation there is a similar delay in the attainment of the maximum response to irradiation resulting from a previously given red impulse (Withrow *et al.*, 42; Virgin, 38, and Figures 7 and 8 in the present paper). This implies that also the effect of light on pigment formation is connected with some time-consuming reactions, probably of the same nature as those involved in the periodic response.

The perception of the photoperiodic response is mainly restricted to green parts, whereby the buds and leaves play the greatest role (Harder *et al.*, 17, 18). The perception of the effects on elongation of the upper internodes of dark-grown pea seedlings seems to be restricted to areas where pigmentation in light first occurs. Such areas are nodes and buds together with developed leaves. Irradiation of the internodes does not give at all the same response (Downs *et al.*, 10; Björn and Virgin, 1). It seems therefore plausible to presume that the red and far-red absorbing pigment system is present in and probably restricted to places in the organism where there are conditions for the formation of chlorophyll pigments.

The most obvious effect of red light on plants is connected with changes of photomorphogenic nature. In connection with these effects many other light-dependent phenomena have been reported with the same action spectrum, among these the pigmentation studied in this present paper. The physiologically active far-red absorbing pigment, resulting from irradiation with red light, has thus a far-reaching effect on the whole metabolism. It may be questioned, however, if such phenomena as reported in this paper are not a secondary manifestation resulting from these general metabolic changes. Other secondary manifestations of a red irradiation should then also be the reported effects on anthocyan formation (Mohr, 30) and pigmentation in tomato cuticle (Piringer *et al.*, 31). Those reactions, which are most important for the organism, and manifested in morphogenic and other far-reaching changes must originate from earlier stages in the reaction chain. Effects on the respiration (Leopold *et al.*, 27; Evenari *et al.*, 12), on IAA-oxidase (Hillman and Galston, 21) and the fact that some of the effects of red light can be brought about by kinetin (Miller, 29; Hillman, 20) and by gibberellic acid (Lockhardt, 28; Vlitos and Meudt, 39) also point in that direction.

Summary

The formation of protochlorophyll and of chlorophyll *a* have been studied under varying irradiations with blue and red light.

Pretreatment with a short impulse of red light followed by darkness for five to six hours results in a disappearance of the lag phase in the formation of chlorophyll *a* during a subsequent continuous irradiation. It could be shown that it is the formation of protochlorophyll which is affected by this treatment. Maximum effect on protochlorophyll formation is obtained after irradiation with low amounts of light (around $9.6 \cdot 10^4$ ergs/cm.²).

The acceleration in chlorophyll *a* formation which is always obtained after a continuous irradiation for two to three hours is the same in blue as in red light under medium intensities of light of the same energy content. This acceleration can be referred to the aforementioned effect on protochlorophyll formation.

Effects of pre-irradiation with blue and red light show that the sensitivity for red as far as the lag phase is concerned is about 22 times greater than for blue light. This means that the difference in sensitivity in respect to the effect on the lag phase will never be manifested when continuous light is used. Such an effect will become obvious only when short light impulse are given followed by a period of darkness prior to a period of continuous irradiation. The consequence of this in connection with photoperiodism is discussed.

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The Influence of Red Light on the Growth of Pea Seedlings. An Attempt to Localize the Perception

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Red light around 660 m μ has a great photomorphogenic effect, particularly on seedlings (3, 6, 7, 8, 10, 11, 14, 15). The effect is partly due to changes in cell divisions and partly to effects on stretching (2, 8). For further references, see Borthwick *et al.* (4, 5). The effect of red light on etiolated *Avena* seedlings consists of a strong inhibition of the growth of the mesocotyl and a promotion of the leaf development (8). In dicotyledons irradiation with red causes a growth inhibition of the lower parts of the plants and a growth promotion of the upper, but the point of reversion can be located in different places: in *Phaseolus* (with epigeic cotyledons) between the hypocotyl and the epicotyl (7, 10, 15), in *Pisum* (with hypogeic cotyledons) two internodes higher up (11, 14).

Goodwin (8) showed that the light inhibition of mesocotyl growth in *Avena* proceeds in two phases: 1. influence upon cell division, which is a very light-sensitive reaction; 2. influence upon the stretching. He also found that if the irradiation is short and of low energy content (4 min.; 0.08 ergs/mm.² sec., administered on the second day after planting, the plants measured on the fifth day) the red light promotes the elongation of the cells of the mesocotyl. Thus the differences between the upper and lower parts of a plant, and between different plants, at least in the second phase, might be more quantitative than qualitative.

The similarity between the action spectrum obtained for this process and such apparently unrelated phenomena as photoperiodism, seed germination,

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pigmentation and others together with the universal inhibition of the red effects by irradiation with far red make it probable that there is one pigment system responsible for all these processes. The nature of this pigment system has been extensively studied and the existence of a reversible pigment transformation seems to be well established (4, 9). The pigment system is present also in albino plants (3).

Earlier attempts to localize the perception in the *Avena* seedling have been made by Araki and Hamada (1), Schneider (12) and Goodwin (8). Araki and Hamada used a spot irradiation of intact plants. They found that both mesocotyl and leaf were influenced by irradiation of coleoptile as well as of mesocotyl. Most sensitive areas were the lower parts of the coleoptile and the upper parts of the mesocotyl, in other words the area around the nodes. Their results, however, are uncertain. With their apparatus they were able to illuminate only three plants at a time. The spectral composition of the light used is not defined, and there is a risk of scattered light. Schneider (12) cultivated excised isolated coleoptile and mesocotyl segments. In both cases the excised plant parts were influenced by red light, the spectral composition of which, however, is not given. In his screening experiments, Goodwin (8) found that whatever part he darkened, the inhibition of the mesocotyl growth was almost as great as if the plants were not screened.

In the case of *Pisum*, Parker *et al.* (11; pp. 202—203) say: "In etiolated pea seedlings the region of radiation perception has not been definitely found nor has an effect involving translocation been established. Although the pea leaf may not be the exclusive organ of perception of the etiolated seedling it seems quite likely that it is involved in perception, as it is also in the photoperiodic control of floral initiation". In an earlier study by Went (14), however, where one leaf of the plant was irradiated while the rest of the plant remained in darkness, he found only a local growth promotion in the illuminated leaf while stem parts and the other leaves were not influenced. It should also be mentioned that in this investigation Went shows that the light has a promoting effect on growth of leaves and stem parts only if the irradiation takes place while growth is still going on. When this has ceased, new growth can not be initiated by irradiation.

In the present investigation studies were begun on *Phaseolus vulgaris* and *Pisum sativum* as to their response to red light. *Pisum sativum* (Svalöfs tordsäkt III, 1956) was finally chosen as experimental material, because the experimental period could be shortened by a few days and there was a greater effect of light on internode length in *Pisum* than in *Phaseolus*. The description and the Tables in the following refer to the above-mentioned *Pisum* material.

Method

A. *Cultivation.* The cultivation technique used in the main experiments was the following: the seeds, previously soaked in a solution of HgCl_2 and formaldehyde, were sorted and only average-sized peas with a smooth seed coat and healthy appearance were used (about 75 per cent were discarded). The seeds were sown in "Vermiculite" moistened with a nutrient solution containing $\text{Ca}(\text{NO}_3)_2$ (1.0 mM), KNO_3 (1.0 mM), MgSO_4 (0.5 mM), KH_2PO_4 (1.0 mM). About ten seeds were sown with the radicula pointing downwards in a circle in round glass vessels (10 cm. in diameter; 5 cm. deep). The seeds were covered by about 1.5 cm. of moisted "Vermiculite". No further nutrient solution was added during the cultivation. The plants were grown in darkness except for the irradiation, at 22°C. The humidity in the cultivation chambers rose to about 100 per cent, but each time a chamber was opened (at least four times a day) it rapidly fell to around 65 per cent. The moisture in the room outside was kept about 45 per cent. In order to get as uniform conditions as possible parallel experiments (in the Tables denoted with the same number) were run in the same chamber with a few exceptions.

B. *Illumination.* The light source was a Phillips filament projection lamp (400 W; 130 V, P. 28, type 6117 c/05) fed by alternating current (120 V). By means of a lens a light beam was obtained which struck the plants sideways. The plants were placed on a clinostat rotating with a speed of one r.p.m. if not otherwise indicated. The lamp was placed outside the darkroom, the light passing through a small opening in the wall with fittings for filters. For orientation in the dark a 15-watt filament lamp was used placed in a lightproof box behind a green gelatine filter (Ilford dark-room safelight G No. 907). According to Parker *et al.* (11) the influence of green light on the plant growth is very slight. In the present case its content of light energy beyond 700 m μ did not appreciably affect the experiments, as in all cases when green light was used, the material had been in darkness for such a long period of time that it could be anticipated that the red-infrared absorbing pigment system was in its red absorbing form (cf. 9).

In the main experiments with red light an interference filter was used with a transmission maximum at 662 m μ (Filtraflex B; No. 1790—9). The intensity of the light beam at its center was approximately 230 ergs/cm.² sec. The irradiation had a duration of 10 minutes unless otherwise indicated.

In order to check the spectral purity of the light its phototropic effect was studied. *Pisum* plants were irradiated without rotation on the fourth and fifth days after sowing. The irradiation had a distinct effect upon the lengths of the internodes. (See Table 1). Repeated observations on the plants at different times did not reveal any

Table 1. *The effect of red light on the growth of dark grown pea seedlings.* Irradiation on 4th and 5th day, plants measured on 10th day. During the irradiation part of the plants were rotated on a clinostat (1 r.p.m.)

Expt.	Treatment	Number of plants	Length in mm. of the different parts					
			Epicotyl	III	IV	V	Leaf 3	Leaf 4
30.	Dark control	9	89	51	9.1	0.2	3.0	—
	Irrad., rotated	9	71	54	90	31	8.1	7.3
	Irrad., not rotated	8	70	48	89	51	8.5	7.5

phototropic bending whatsoever. For comparison coleoptiles of *Avena* were also irradiated. Thirtyone 4-day-old plants were irradiated once. At the age of six days the mesocotyl averaged 26 mm. compared to 37 for 22 dark controls. In other similar experiment the values 22 and 38, respectively, were obtained. In the irradiated plants the leaf had broken through the coleoptile in 21 cases out of 31, in the dark control in no case. Phototropic bending could not be established.

C. *Measurement.* The lengths of internodes and leaves in the Tables are expressed in millimeters. The leaf length is given as the distance between the tip of the straightened lateral lobe and the point where the petiole departs from the stem, measured along the dorsal side. In the internodes the distance between the above-mentioned points at adjacent nodes was measured. The values are given to the nearest whole millimeter, the averages calculated usually with an exactitude of two figures. In some cases the values for the epicotyles are somewhat uncertain, as measurements were carried out on intact plants when not all cotyledons had yet reached the surface of the plant bed. Such values are placed in brackets. The standard deviation given

for some experimental values is calculated according to the formula $\sqrt{\frac{\sum x^2}{n} - \bar{x}^2}$

(x denoting individual values, \bar{x} their average, and n the number of values). The internodes are in the Tables numbered with the hypocotyl as No. I and are denoted in Roman figures. The leaves are numbered in Arabic figures with the one between the epicotyl and internode III as No. 1. All experiments are numbered in the order they were started. Times are expressed as number of days from sowing.

D. *Screening.* In order to localize the light perception, different parts of the plants were screened with black paper or (starting with Experiment 31) aluminum foil. Apical screens were removed after each irradiation so that growth could continue freely. At the first irradiation the cotyledons were still below the "Vermiculite" surface, but at harvest they had usually reached it. Consequently the cotyledons were partly irradiated.

Removal of plant parts in Experiments 37 and 38 (Table 6) were made with a pair of tweezers.

Experimental Results

A. *Dark controls.* In most cases the plants were harvested and measured on the tenth day after sowing. In the cases when two values are given for the number of measurements the higher figure stands for the lower part of the plant and the other for the upper part. The reason for giving two figures is that some plants have been broken or otherwise damaged so late during the course of the experiment that this is not assumed to have influenced the lengths of the lower internodes.

It can be seen from the Tables that the values for the epicotyls are higher in the later experimental series as compared to those with lower experimental numbers. The reason for this is probably that starting with experiment 30 a new batch of seeds were used. In the older material the values are between 65 and 88 mm. (average 74), in the new ones between 86 and 95 (average 90). The latter values seem to have a smaller variance. This is illustrated in

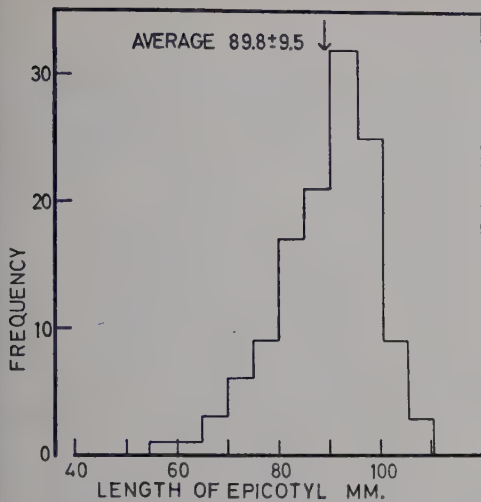


Fig. 1.

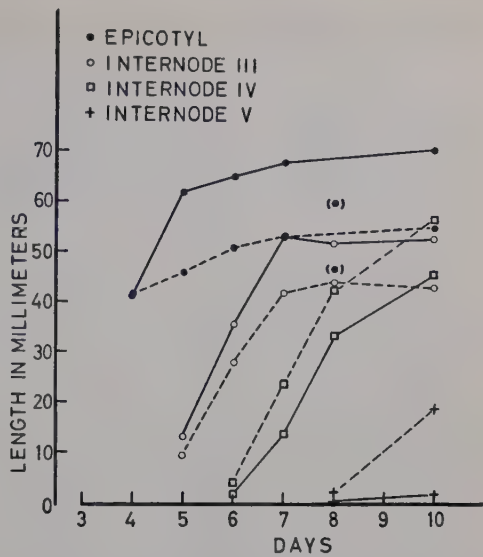


Fig. 2.

Figure 1. *Distribution of epicotyl lengths of 10 days old dark-grown pea seedlings. (Expts 30—39; 127 plants in total.)*

Figure 2. *Growth of different internodes of dark-grown (————) and of irradiated (-----) pea seedlings. Values mainly from Expt 20.*

Figure 1 which shows the distribution of all epicotyl lengths in the latter group. The variance is greater in the higher internodes, which is natural, since these are growing more rapidly and a small difference in the stage of development causes a great deviation in length. This can be seen in Figure 2. The values presented are obtained from Experiment 20 (Table 3) except for

Table 2. *The effect of red light on the growth of dark grown seedlings. The plants have been irradiated on the 4th to the 8th day after planting (10 min. a day) and are measured on the 10th day. Figures show increase in mm. over dark controls.*

Expt.	Number of plants	Epicotyl	III	IV	V	Entire stem	Leaf 3	Leaf 4
20 R	9	—16	—10	+11	+17	+2	+4.1	+3.6
28 R	6	—20	—26	+35	+30	+19	+5.7	+3.6
31 R	8	—27	—6	+42	+5.9	+15	+4.9	—
33 R	11	—24	+2	+47	+21	+46	+3.9	—
35 R	10	—24	—12	+26	+9.1	—1	+2.6	—
36 R	11	—29	—14	+58	+32	+47	+5.5	—
37 R	10	—19	—14	+46	+40	+53	+6.2	—
39 R	10	—27	—14	+26	+34	+19	+5.3	+5.9
Averages		—23	—12	+36	+24	+25	+4.7	

Table 3. *The effect of red light on the growth of dark grown pea seedlings when the epicotyl (internode II) is screened. M: dark controls, R: wholly irradiated, A: screened and irradiated.*

Expt.	Number of plants	Age (days)		Length in mm. of the different parts					
		at irradiation	at measurments	Epicotyl	III	IV	V	Leaf 3	Leaf 4
20 M	10	—	5	(62)	13	—	—	—	—
20 R	9	4; 5	5	(46)	9.6	—	—	—	—
20 A	11	4; 5	5	(48)	9.1	—	—	—	—
20 M	10	—	6	(65)	36	2.3	—	4.5	—
20 R	9	4; 5	6	(51)	28	3.9	—	6.6	—
20 A	11	4; 5	6	(53)	28	2.0	—	4.9	—
20 M	10	—	7	(68)	53	14	—	4.4	—
20 R	9	4; 5; 6	7	(53)	42	24	—	8.0	—
20 A	11	4; 5; 6	7	(52)	44	21	—	7.4	—
20 M	10	—	8	(60)	52	34	—	4.6	—
20 R	9	4; 5; 6; 7	8	(47)	44	43	2	7.8	6.8
20 A	11	4; 5; 6; 7	8	(48)	46	39	0.5	6.8	5.1
20 M	9	—	10	71	53	46	1.8	4.3	4.0
20 R	9	4; 5; 6; 7; 8	10	55	43	57	19	8.4	7.6
20 A	11	4; 5; 6; 7; 8	10	53	46	74	26	6.1	9.4
22 M	12	—	9	74	60	12	—	2.8	—
22 R	12	4; 5; 6	9	54	47	71	4.5	5.4	5.3
22 A	11	4; 5; 6	9	53	45	61	3.0	5.4	4.7

the first value for the epicotyl (42 mm.) which is taken from another series of measurements of 31 plants — not harvested prior to measuring. The absolute values are not very typical but the course of development is characteristic. It was generally found that the conformance between experiments simultaneously performed (having the same number) is better than the conformance with other experiments. Therefore values from irradiated plants have always been compared with simultaneously performed dark controls instead of be-

Table 4. *The effect of red light on the growth of dark grown pea seedlings when everything above the epicotyl is screened. Irradiation on 4th, 5th, 6th, 7th, and 8th day. The plants were measured on 10th day after planting.*

Expt.	Treatment	Number of plants	Length in mm. of the different parts					
			Epicotyl	III	IV	V	Leaf 3	Leaf 4
35.	Dark control	9	90	61	9.3	—	2.6	—
	Dark control	6	95	53	3.7	—	2.5	—
	Wholly irradiated	10	69	45	33	9.1	5.2	—
	Screened	4	74	45	35	5.6	7.0	—
39.	Dark control	9—11	92	66	33	0.3	3.5	4.2
	Dark control	10	91	59	43	1.9	4.5	5.9
	Wholly irradiated	10	65 ± 10	49	64	35	9.3	11
	Screened	7	75	47	71	26	7.6	7.6

Table 5. *The effect of red light upon the growth of dark grown pea seedlings when varying parts of the plant has been screened. Irradiation on 4th, 5th, 6th, 7th, and 8th day after planting. Plants measured on 10th day.*

Expt.	Treatment	Number of plants	Length in mm. of the different parts					
			Epicotyl	III	IV	V	Leaf 3	Leaf 4
25.	Dark control	19	72 \pm 9	68	42	—	4.0	—
	Wholly irradiated	5	59 \pm 2	56	65	0.6	5.2	—
	Screened:							
	10 mm. of the tip	10	70 \pm 8	62	68	0.5	4.3	—
28.	Dark control	10	86	81	51	1.7	4.5	3.7
	Dark control	7—8	86	79	38	1.1	4.1	3.0
	Wholly irradiated	6	66	54	80	31	10	7.0
	Screened:							
	10 mm. of the tip	6	79	54	67	24	7.0	8.2
31.	Dark control	10	86	50	3.3	—	2.2	—
	Wholly irradiated	8	59	44	45	5.9	7.1	6.0
	Screened:							
	10 mm. of the tip	6	64	42	14	—	0.4	—
	Everything except apical 10 mm.	5	60	41	34	3.6	5.4	4.2
33.	Dark control	10	93	42	6.3	—	2.2	—
	Dark control	9	86	45	9.2	—	2.2	—
	Wholly irradiated	11	66	46	55	21	6.1	6.0
	Screened:							
	See footnote 1	5	64	45	59	15	6.4	6.4
	Leaves 3 a. 4	3	70	40	43	11	8.0	4.8
	Everything except leaves 3 a. 4	7	62	45	41	8.0	6.7	5.7
36.	Dark control	8	91	53	18	0.4	3.3	—
	Dark control	8	89	66	24	—	3.6	—
	Wholly irradiated	11	61	46	79	32	9.0	9.1
	Screened:							
	The nodes	5	65	44	41	7.2	6.6	5.0
	The internodes	5	66	45	54	14	7.6	—
39.	Dark control	9—11	92	66	33	0.3	3.5	4.2
	Dark control	10	91	59	43	1.9	4.5	5.9
	Wholly irradiated	10	65 \pm 10	49	64	35	9.3	11
	Screened:							
	See footnote 2	7	75	47	71	26	7.6	7.6
	" " 3	9	76 \pm 9	46	63	19	6.0	7.1
	" " 4	11	73	43	66	22	7.3	7.9

¹ During the first irradiation 10 mm. below the free tip (2 mm.) was screened; when the plants became older and a plumular hook had developed, 10 mm. below the tip of the hook was screened, while the down-hanging part was left uncovered.

² Everything above the epicotyl (including node 1).

³ 2 mm. above and 8 mm. below the nodes.

⁴ 8 mm. above and 2 mm. below the nodes.

ing compared with a total average. When two dark controls have been made simultaneously, comparison has been made with their average.

B. *Red irradiation of whole plants.* The effect of irradiation with red without screening or removal of any part of the plant is seen in Table 2 and in

Table 6. *The effect of red light on the growth of dark grown pea seedlings when varying parts of the seedling have been removed. Irradiation on 4th, 5th, 6th, 7th and 8th day in Expt. 37; on 5th, 6th, 7th, and 8th day in Expt. 38. Plants measured on 10th day.*

Expt.	Treatment	Number of plants	Length in mm of the different parts					
			Epicotyl	III	IV	V	Leaf 3	Leaf 4
37.	Dark control	9	87	63	36	—	3.1	—
	Dark control	9	91	59	18	0.7	3.9	—
	Wholly irradiated	10	70	47	73	40	9.7	9.6
	<i>Removed parts:</i>							
	The leaves	8	67	48	75	24	—	—
38.	Dark control	10	90	51	9	—	2.5	—
	Dark control	9	93	71	25	0.6	2.9	—
	Wholly irradiated	11	82	43	51	9	7.1	5.9
	<i>Removed parts:</i>							
	Everything above internode III (dark)	12	84	66 ± 14	—	—	—	—
	Everything above internode III (light)	10	86	44 ± 3	—	—	—	—

Figure 2. The light has an inhibiting effect on the epicotyl and internode III but the opposite effect on the higher internodes and on the leaves. This holds for all examined periods of irradiation and measurements.

C. *Screenings and excisions.* The result of screening the epicotyl during irradiation is shown in Table 3. If everything above the epicotyl is screened, the values shown in Table 4 are obtained. The values from other screening experiments are shown in Table 5. They will, like the results from the excision experiments (Table 6) be discussed below.

Discussion of the Results

As can be seen from the Tables, no screening completely nullified the "red effect". Whatever plant part was screened, the screened plants were more similar to the completely irradiated plants than to the dark controls. This might be caused by ineffective screening, as it was not possible to wrap the paper and foil too tightly without injuring the plants. Also the transparency of the plant material caused another difficulty. *However, in none of the series of screening experiments, the screened plants are as similar to the completely irradiated plants as those where only the epicotyl was screened.* If everything except the epicotyl is screened, however, the plants become a little more similar to the dark controls. This is not only due to the fact that the screenings were quantitatively different, which is shown by Experiments 36 and 39 (Table 5). Here it can be seen that small screenings may have as great an effect as big ones. *Therefore one can draw the conclusion that the epicotyl*

is less sensitive to light than other parts. In Experiment 28 the epicotyl of the screened plants has approached the value for the dark control, and the other internodes conform with the completely irradiated plants. This might be explained in the following way. In the first irradiation, when the epicotyl is influenced, the apical screening covered everything except the epicotyl but later on when the epicotyl was full grown, the light-sensitive parts were exposed. However the variance of the values is great. Experiment 25, which shows the same result cannot be explained in this way. In Experiments 31 and 33 there are no distinct differences at all, probably because the screens have not been tight enough. Experiment 37 (Table 6) shows that not only the leaves percept the light. It is rather difficult to remove everything of the youngest leaves without injuring the top bud, but no leaf was longer than two millimeters when irradiated. It is of interest to notice that the leaves of the pea plants were yellow at the time of harvest, while the rest of the plants were more or less colourless. After some time in daylight the leaves were also the first parts to become green. They also turned green sooner in plants which had been previously treated with red light than in dark controls. This is in conformance with the effect of red light on chlorophyll formation studied on other plant material (13, 16).

From the values obtained in Experiment 36 (Table 5) the conclusion may thus be drawn that the nodes are more sensitive organs of perception than the internodes. When the nodes were screened (in the last irradiation about 20 per cent of the stem was screened) the effect was as great as when the internodes (80—90 per cent of the stem covered in the last irradiation) was screened. Experiment 39 (Table 5) points in the same direction: screening of the nodes had the same effect as screening of both nodes and internodes.

Amputation of the stem (Experiment 38, Table 6) was too serious an injury to the plant to make any conclusion of light effects possible. The difference in length of internode III between the two groups of amputated plants may appear to be great, but this can be shown to be due to chance.

The conclusions from the experimental results discussed above may thus be summarized: There are no *distinct* areas of perception in the case of photomorphogenic changes caused by red light. There are areas, however, which are more light-sensitive than others. These areas seem to agree well with the distribution of chlorophyll pigments, carotenoids and their precursors in the plant. Nodes are thus more sensitive than internodes. The findings reported in this paper thus fully support those obtained by Araki and Hamada (1) for *Avena* seedlings. Also in this plant the nodes are the areas most sensitive to red light. The fact that red light with the same spectral composition as used in the present experiments has a distinct effect on chlorophyll formation — at least in the earlier stages — (13, 14), and that

the effect is nullified by infrared light (16) indicate that the responsible pigment mechanism is located in the places where the other plant pigments are to be found. If this conclusion is correct, no red-infrared responses should be obtained in plant parts where no chlorophyll or carotenoids can be formed. This statement may seem to disagree with the fact that also albino plants show red responses (3). The lack of chlorophyll synthesis, however, does not exclude the existence of earlier stages in the pigment formation. According to Borthwick *et al.* (3) the pigment responsible for the red-infrared absorption may be an open tetrapyrrole. Whether the compound in question is a link in the reaction process leading to chlorophylls and therefore present in places where chlorophyll synthesis takes place or a separate compound unrelated to chlorophylls is an open question.

Summary

Dark-grown pea seedlings have been irradiated for short periods (10 min. a day) beginning with the fourth day after planting. The light used had its maximum energy around 660 mμ.

The effect of the irradiation on unscreened plants consists of a decrease in the lengths of the epicotyl (first internode), previously reported on many occasions, an increase in the length of the internodes higher up, and also of an increase in the development of the leaves.

By screening different parts of the seedling during irradiation, the effect of the red light varies depending on which parts are exposed to light. There seem to be no distinctly definable areas of radiation perception. The nodes, however, are more light-sensitive than the internodes. The distribution of the areas giving the strongest response to red irradiation seem to agree with the sites for the formation of the green and yellow pigments of the plant.

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The Effect of Neutron Treatment of Seeds on Seedlings of Diploid and Tetraploid Red Clover (*Trifolium pratense*)

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Material and Methods

Experiments have been carried out in 1954 (I), 1955 (II) and 1956 (III) on the development of diploid and tetraploid red clover seedlings after neutron treatment of dry, dormant seeds.

The diploid material included six strains:

1. Molstad, local strain of late red clover: Experiment I and II+germination test.
2. SF 4410, commercial seed of late red clover: Experiment III.
3. SF 3645, " " " " " " " " III.
4. SF 2280, " " " " " " " " Germination test.
5. SF 4409, " " " " " " " " "
6. Vidarshov II, bred strain of late red clover: Experiment I and II+germination test.

All these strains are closely related types.

The tetraploid strains consisted of various selections produced at the Institute of Genetics and Plant Breeding, on the basis of plants from various strains of late red clover:

7. RT 1: Experiment III.
8. RT 20: " III.
9. RT B1: " II+germination test.
10. RTE 5: " I, II " "
11. RT 606: " I.

None of these tetraploid strains represents a doubled form of any of the diploid strains tested. They should represent, however, a fairly representative sample of strains of Norwegian late red clover which have appeared in our test to be very similar in morphological and physiological characters. In the following, 2x and 4x are used to designate diploid and tetraploid forms. The methods of radiation treat-

ment are those described by Mikaelson and Aastveit (8). The seeds were placed in cylindrical aluminium tubes and irradiated in the centre of the heavy water reactor, *Jeep*, which is run by *The Joint Establishment of Nuclear Research* at Kjeller, Norway.

In the centre of the pile there are thermal, epithermal and fast neutrons. Detailed descriptions and definitions of the radiations in this position are given elsewhere (2, 9).

The reactor was operated at 10 KW. The main part of the radiations consists of thermal neutrons. The seeds received the following doses of thermal neutrons, 0, 1, 2, 3, 4, 6, 8, 9, and 10×10^{12} n_{th}/cm^2 . From Ehrenberg and Sælends (2, 3) calculations, however, the lesser dose of non-thermal neutrons is responsible for 70—80 per cent of the total radiation dose. The radiation doses are determined by chemical dosimetric methods (3). According to these measurements the seeds received the following doses: 600, 1200, 1800, 2400, 3600, 4800, 5400 and 6000 *rep*.

The contaminating gamma radiation is neglected since it will give an unimportant contribution to the total radiation dose.

The seeds were sown in boxes in the greenhouse.

Experiment I was of a preliminary nature without any replications. In Experiment II we used a split plot design with 5 replications of large randomized treatment plots, divided in 4 randomized subplots for strains. Fourty seeds were sown per subplot. The four strains used in Experiment II were treated anew and a germination test made at The State Seed Testing Station. At the same laboratory six strains were tested in 1956. Four of these, No. 2, 3, 7, and 8 were sown in boxes in the greenhouse, in the same way as in Experiment II, but with 25 seeds per subplot (Experiment III). In the following the effect of the treatments on germination and some seedling characters will be discussed.

Germination Percentage

Germination capacity was determined in all the experiments. In the greenhouse sowings the number of seedlings emerged above the soil were counted at three dates. The germination percentages were based on the number of emerged seedlings ten days after sowing. The material in Experiments II and III was also tested for germination at The State Seed Testing Station. *On the average the neutron treatments had no significant effect on germination.* In the greenhouse sowings a significant increase in germination percentage after treatment was found in single cases, but no such effects were observed in the sowings at The State Seed Testing Station of the same material. Our general results agree with those obtained in other species, *viz.*, alfalfa (1), barley (7, 9), oats and wheat (4).

Rate of Germination

At The State Seed Testing Station the number of sprouted seeds was counted after 4 and 10 days. The results from these tests and from the green-

house sowings give no evidence of an effect of neutron treatment on the rate of germination. In Experiments II and III the tetraploid seedlings had a more rapid emergence than the diploids, while no difference in germination rate was observed in the tests at The State Seed Testing Station, in which the seeds were germinated on filter paper. *It must be concluded that no difference has been found between diploids and tetraploids in true rate of germination, but that the latter showed a more rapid emergence in soil sowings.* These results are in agreement with our experience in red clover that the tetraploids generally have given uniform stands of vigorous seedlings, a fact which may probably be ascribed to the larger seed size.

Chlorosis

On the treated seedlings white, yellowish white, and yellow spots and streaks appeared on the first leaves. The cotyledons were always normal green. In Experiment II, sown on February 8, no chlorosis was observed until after March 1. The chlorosis was found mainly on the first 2—3 leaves, the later leaves usually being normal green. The mean percentages of chlorotic seedlings for the diploid and tetraploid groups of strains are given in Table 1. It is evident that the tetraploids have a smaller percentage of chlorotic seedlings. The interaction between polyploidy and treatment was significant at the 5 % level, $F.1/60=2.515^*$ in Experiment II and at the 0.1 % level in Experiment III, $F.1/48=18.744^{***}$. The chlorotic seedlings in Experiment II were divided in 3 classes according to the extent of chlorotic spots. The classification showed that chlorosis was more marked in the diploids than in the tetraploids.

A classification of chlorosis on the third and later leaves was made in Experiment II on March 31.:

		% of chlorotic seedlings:				
Dosage.....	0	1	3	6	9	
Diploids	0.5	0.8	2.8	3.6	6.9	
Tetraploids	0.5	0.5	0.5	4.0	6.5	

The classification shows that only a few of the third and later developing leaves showed chlorosis, and after some further time all new leaves were entirely green.

Davis and Hammons (1) observed different types of chlorosis in Ranger alfalfa seedlings after X-ray treatment. This is the only reference found of chlorosis after treatment of ionizing radiations. In most species no such effect has been observed. The short duration of the chlorosis seems to indicate that it has been caused by damage of the radiation on cytoplasmatic

Table 1. *Percentage of chlorotic seedlings in 2x and 4x red clover after neutron treatment of the seeds.*

Treatment dose: ($\times 10^{12}$ n _{th} /cm ²)	0	1	2	3	6	9
Experiment I. Sown Oct. 2, classified Nov. 11, 1954						
2x, 2 strains	0	62.5	96.5	80.9	100.0	—
4x, 2 strains	0	0	—	63.9	74.3	—
Experiment II. Sown Febr. 8, classified March 12, 1955						
2x, 2 strains	0	2.1	—	5.7	67.2	96.6
4x, 2 strains	0.8	2.5	—	10.0	60.3	89.5
Experiment III. Sown March 19, classified April 3, 1956						
2x, 2 strains	0	—	—	77.5	96.7	100.0
4x, 2 strains	2.3	—	—	25.5	87.8	100.0

constituents. These constituents may have been the leucoplasts or some substance necessary for transformation of the leucoplasts into chloroplasts.

If one assumes that the chlorosis is due to some damage on the plastids, the difference found must be due to some differences in the plastid constitution in the diploid and tetraploid forms, such as the number and size, or the qualitative characters of the plastids. Some studies have been made on the chlorophyll content of the polyploids. The comparisons between diploids and polyploids have been based partly on artificially produced autopolyploids, partly on auto- or allopolyploid varieties within a species or species within a genus. In *Bryum caespiticum* v. Wettstein (11) found an increase in number, but no increase in size of chloroplasts by chromosome doubling in mosses. Most other authors, e.g., Heilbronn (5), Pirschle (10), and Levan (6) have found a lower chlorophyll content per fresh weight unit in the tetraploids. In a clone of *Trifolium pratense* in the material of Levan only a slight difference between the diploids and the tetraploids was found. Levan also found a smaller content of chlorophyll per gram dry matter in the tetraploids. Studies of the chlorophyll content per dm² leaf surface have given variable results. In 3x and 4x forms of three species of *Epilobium*, in 4x and 8x forms of *Torenia fourniera*, and in 2x and 4x forms of *Antirrhinum majus*, Pirschle (10) found a higher chlorophyll content per dm² leaf surface in the polyploids than in the diploids. In two varieties of *Impatiens balsamina* a slightly lower content was found in the tetraploids. Levan (6) concluded that it is not possible to frame a general rule for the behaviour of pigment production of polyploids. These results cannot give any clue to the difference in chlorosis between diploids and tetraploids found in our experiments. Only a detailed analysis of the properties of chlorophyll in the treated forms might give such a clue. Such a study might also give an explanation of the fact that the cotyle-

dons showed no sign of chlorosis. The tentative explanation may be ventured that the plastids were differentially developed in the cells of the embryo, the plastids in the cells giving rise to the first and second leaves being in a sensitive stage, which may have been passed in the cotyledon cells.

Deformed Leaves

The seedlings from treated seeds showed various amounts of deformed leaves. The cotyledons were normal, but the first, second and third leaves showed deformations, the later leaves being in most cases normal. The deformations were of various kinds: strong wrinkling, indentations of leaf edges, uni- and bifoliate instead of trifoliate leaves. The number of seedlings with deformed leaves were counted and the degree of deformation classified as little, medium and heavy. The average percentages of seedlings with deformed leaves for the diploid and tetraploid groups are given in Table 2.

Table 2. *Percentage of seedlings with deformed leaves in 2x and 4x red clover after neutron treatment of the seeds.*

Treatment dose: ($\times 10^{12}$ n _{th} /cm ²)	0	1	3	6	9
Experiment I.					
2x, 2 strains	0	0	8.1	49.8	—
4x, 2 strains	1.2	2.9	9.2	58.0	—
Experiment II.					
2x, 2 strains	5.7	21.0	80.2	88.6	96.6
4x, 2 strains	4.8	4.4	27.5	77.9	87.9
Experiment III.					
2x, 2 strains	0	—	7.7	84.0	97.0
4x, 2 strains	0	—	0.7	33.6	76.6

Table 3. *The effect of neutron treatment of seeds of 2x and 4x red clover on seedling vigour. % of weak plants.*

Treatment dose: ($\times 10^{12}$ n _{th} /cm ²)	0	1	2	3	6	8	9	10
Experiment I.								
2x, 2 strains	16.5	24.3	21.2	46.7	96.7	100.0	—	100.0
4x, 2 strains	11.8	19.5	—	24.0	30.4	71.5	—	86.7
Experiment II.								
2x, 2 strains	37.7	39.7	—	44.3	65.1	—	92.2	—
4x, 2 strains	33.5	19.4	—	20.8	40.8	—	56.0	—
Experiment III.								
2x, 2 strains	18.2	—	—	30.0	84.4	—	89.4	—
4x, 2 strains	6.7	—	—	7.6	37.5	—	57.0	—

In all three experiments we found a significant effect of treatments on the percentage of deformed leaves. With increased dosages we found also an increase in the degree of deformation. In Experiments II and III, but not in Experiment I, a significant interaction between treatment and polyploidy group was found, $F_{1/60}=29.827^{***}$ and $F_{1/48}=20.822^{***}$ respectively. Also in this character the tetraploids show a greater resistance against injuries from neutron radiation than the diploids.

Davis and Hammons (1) found on alfalfa seedlings from seed treated with x-rays that doses above 42 600 r reduced materially the formation of the unifoliate leaf. Irregular leaf shape, wrinkling and serrations were observed in the first trifoliate leaf. A dose of 31 900 r or more was necessary to materially effect this leaf.

Vigour of Seedlings

A visual estimation was made of the vigour of the seedlings, separating them in Experiments I and II in 5 classes, and in Experiment III in 4 classes. In Table 3 are given the average percentages of the two lowest classes combined ("very weak" and "weak") for the diploid and tetraploid groups of strains. The tetraploid seedlings are more vigorous than the diploid in all three experiments. For additional information the total distribution in four vigour *classes* may be cited for all treatments in Experiment III ("weak" to "very strong"):

Class	1	2	3	4
2x	7	49	28	2
4x	4	21	50	17

There is clear evidence that the treatments have given an increase in percentage of weak plants. The data in Table 3 further indicate that the percentage of weak plants has been increased relatively more in the diploids.

In Experiment I the diploid seedlings from the largest dose treatment were very abnormal and died at the cotyledon stage, while the tetraploid seedlings were fairly normal and able to continue growth.

Weight of Seedlings

In Experiment II the fresh weight and in Experiment III the fresh weight and dry matter per plot were determined and the weight per plant calculated from the number of plants per plot (Table 4). In Experiment III no effect of treatment on the *percentage of dry matter* was found:

Dosages	0	3	6	9
2 diploid strains	11.2	11.5	11.4	11.2
2 tetraploid strains	9.7	9.9	9.8	9.7

Table 4. *Effect of neutron treatment of seeds on seedling weight in 2x and 4x red clover.*

Material	g. fresh weight per plot					g. fresh weight per plant				
	0	1	3	6	9	0	1	3	6	9
Experiment II.										
2x, 2 strains	28.1	33.2	33.3	28.9	24.2	1.46	1.48	1.39	1.17	0.96
Rel. 100	100	118	119	103	86	100	101	95	80	66
4x, 2 strains	39.6	45.5	40.2	38.0	27.8	1.97	2.45	2.11	1.93	1.52
Rel. 100	100	115	102	96	70	100	124	107	98	77
Experiment III.	mg. dry weight per plot:					mg. dry weight per plant:				
2x, 2 strains	682	—	690	491	472	51	—	48	36	35
Rel. 100	100	—	101	72	69	100	—	94	71	70
4x, 2 strains	1115	—	1052	888	694	68	—	68	62	52
Rel. 100	100	—	94	80	62	100	—	100	91	76

In conformity with earlier results the tetraploids have a lower dry matter content. A negative correlation was found between number of plants per plot and weight per plant, but the effect was small, the regression coefficient being: $b=0.005$ g per plant. The tetraploids in both experiments have a higher weight per plant than the diploids, in agreement with the classification for vigour. The analyses show a significant effect of treatments, in Experiment II $F.4/16=6.082^{**}$ and in Experiment III $F.3/12=30.881^{***}$. The data in Table 4 indicate that the smallest doses, 1 and 3, have had little effect on weight, and a calculation of the treatment components in both experiments shows that the main treatment effect is due to the two largest doses (6×10^{12} and 9×10^{12}). This may indicate a threshold effect. In Experiment II there is no significant interaction between polyploidy groups and treatment, while in Experiment III there is a significant interaction, $F.1/48=4.844^{**}$. The data of Experiment II, although not significant, point in the same direction as those of Experiment III, showing that the tetraploids have suffered a smaller reduction in plant weight from the treatments than the diploids.

Summary

1. Neutron treatment of dry dormant seeds of diploid and tetraploid red clover strains has shown little effect on germination. In a few cases an increase in germination percentage was found after treatment.
2. No effect of treatment on rate of germination was found. The tetraploid strains showed a more rapid emergence than the diploids in soil sowings, while no such difference was found in germination tests on filter paper in the laboratory.

3. Neutron treatment caused the appearance of chlorosis in the 1st, 2nd and 3d seedling leaves, but never in the cotyledons. The later leaves usually showed no chlorosis. The tetraploids showed a smaller degree of chlorosis than the diploids, and thus a greater resistance against this type of radiation damage.
4. The seedlings from treated seeds showed various amounts of deformed leaves. The cotyledons and usually the fourth and later leaves were normal. The tetraploids showed a greater resistance to leaf deformation, caused by radiation, than the diploids.
5. The short duration of chlorosis and leaf deformation indicates that these effects are due to extragenic changes.
6. Tetraploid seedlings had significantly higher weights per plot and per plant than the diploids. The treatments caused a significant reduction in plant weight, this effect being mainly due to the two largest doses of 3600 and 5400 rep. In two experiments the data indicate that the tetraploids suffered less reduction in plant weight than the diploids, but the interaction polyploidy groups — treatment was significant only in one of them.

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Passive Components in the Ion Absorption of the Plant II. The Zonal Water Flow, Ion Passage, and Pore Size in Roots of *Vicia Faba*

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Introduction

The conception that the ion uptake of the intact plant is of a complex nature has been widely accepted today. The following have been distinguished as components of the ion uptake:

(A) *Passive* equilibration through *diffusion* between the nutrient solution and the free space of the root. Depending upon the ion species subsequent *adsorption* to electrically charged points in the cell walls and plasma may be more or less important;

(B) *Active accumulation* in the plasma and to the vacuole of the root cell including;

(C) *Actively initiated* transport of salt and water through *bleeding* from root to shoot;

(D) *Passive mass movement* of ions with the transpiration stream.

(E) In the shoot there is also *diffusion* with *adsorption* and *accumulation* corresponding to that in the root (*i.e.*, A and B).

Surveys of the ion absorption of the plant have been given by Hylmö (1953, 1955), Broyer (1956 a, b), Burström (1957), Epstein (1956 a, b), Kramer (1956 a, b), Kylin and Hylmö (1957), and Briggs and Robertson (1957).

It has been known for a long time that in isolated roots, when the first rapid equilibrium through diffusion (A) in the free space is reached, the principal ion absorption occurs in the tip of the root. This ion absorption

is apparently due to accumulation (B) in the cytoplasm and, principally, to the vacuole of immature cells in the zones of cell division and enlargement. As shown with P^{32} by Wiebe and Kramer (1954) and Kramer (1956 a, b) in intact barley plants, there is also the passive passage of ions from the medium to the shoot (D). This later ion absorption occurs principally in the older parts of the root, where the xylem is already completely differentiated. Endodermis apparently does not stop the passage either of water or of salt.

One detail in the ion uptake of the plant, which is at present the subject of discussion, is whether the ion transport linked with the water flow (D) is really passive. This is maintained by Hylmö (1953, 1955) and recently by Epstein (1956 b), Kramer (1956 b) and Kylin and Hylmö (1957). The ions are considered to be passaged from the medium with the transpiration stream through the root and further to the shoot without being involved in the metabolism of the root.

A smaller part of this water linked ion transport is, however, due to bleeding. The intensity of the bleeding is probably increased, at least in certain cases, by the transpiration stream. Ions which originally were actively accumulated by the xylem are later passively carried along with the transpiration stream. In this way the concentration of the ions in the xylem is reduced, with the result that the ionic equilibrium is deranged in favour of a renewed accumulation in these punctured cells (Hylmö 1953). This bleeding component (C) in the ion transport is thus involved during the transport through the root in the metabolism there. The principal part of the ions in contradiction of this is pasaged in the mass flow all the way from the medium through the root to the shoot. Passive transport of ion as mass movement has been shown for calcium, chloride, bromide, phosphate, and sulphate.

For ions like potassium and nitrate which are accumulated very rapidly and in a high concentration in the vacuoles we have to suppose a dominating bleeding component in the ion transport to the shoot (Hylmö 1953, Broyer 1956 b).

Several new investigations give evidence of the fact that ions and molecules without being involved in the metabolism of the root, are passaged from the medium to the shoot in the free space of the root. Epstein (1956 b) conclude that in 6-day old barley seedlings, the shoot is not *in series* with the active transport mechanism of the root cells, but *in parallel* with it. Epstein demonstrated that precisely those sulphate ions not actively accumulated by the root cells reached the shoots bypassing the actively absorbed ions.

In ripening plants of wheat Frazier *et al.* (1956) showed that P^{32} flux directly from the soil to the seed without being involved in the metabolism of the root. P^{32} was transported with a speed of at least 64 cm. an hour. Also the transpiration research with sunflower plants of Wright and Barton (1955) favours the opinion of a passive transport of radioactive phosphate to the shoot.

It is probably unwise to press comparisons between the uptake of ions and the uptake of neutral substances such as griseofulvin or organic radicals like acetate, but the fact that the absorption of large molecules as antibiotics is direct proportional to the flow of water (Crowdy *et al.* 1956) favours the opinion that there is no hindrance for the passage through the root. The solutions could freely be drawn

in the free space of the root from the medium to the shoot. Also the fact that the influx coefficient for griseofulvin calculated from the data of Crowdy *et al.* (1956) is of the same order as for ions argues in favour of the same mechanism of dilution for ions and neutral molecules. The influx coefficient is an expression of the concentration of the true transpiration stream in relation to the concentration of the medium (Hylmö 1953, 1955). Thus solutions of antibiotics are also diluted when passing the root. Drugs of sulphonamide type are also passing to the plant in proportion to the water flow (Crowdy and Jones 1956). In the *Phaseolus* plant, as has been shown by Koozin *et al.* (1956) labelled acetate ions are taken up in direct proportion to the waterflow (deuterium).

The idea that ions can pass through the root without being involved in the metabolism is in contradiction to the symplast hypothesis of the Groningen school, according to which all ions are actively absorbed by the symplast in the epidermal and cortical cells and are then transported in the plasm to the stele (Arisz 1956).

In a recently published paper of the Groningen school Brouwer (1956) has reanalyzed the water linked ion component. Whereas Brouwer (1953, 1954) earlier had maintained that the entire increased ion uptake with increased water flow was active, he concluded in his latest paper that the main part of the water linked component is dependent on the metabolism of the living root cells but that a lesser part is passive. In this way the difference in the comprehension of this question between Brouwer (1953, 1954) and Hylmö (1955) has been brought on a quantitative level. The interpretations in these papers are strongly divergent as to the relative amounts of the active and passive components. Hylmö (1953, 1955) considers the active part of the transpiration component of the chloride uptake to be only very insignificant and to represent increased bleeding. Brouwer (1953, 1954, 1956) believes on the other hand, that the active component in the increased ionic transport with increased water flow is dominating and linked to a mechanically induced change in "conductivity" of the root for ions as well as for water.

With regard to the central role Brouwer's interpretation has been accorded in the symplasm theory for the transport in the root (Arisz 1956), the author has deemed it advisable for a second time to reexamine the results of Brouwer. Only the conception "conductivity" and the relation between "conductivity" and the ion uptake will be treated here. The other results, among others with inhibitors and with displacements in the value of the influx coefficient, no matter how interesting, will be discussed first in another connexion.

It will be pointed out that new investigations with inhibitors confirm Hylmö's (1955) earlier reinterpreting of the data of Brouwer for the connexion between water flow and ion uptake with and without inhibitors (DNP, KCN, N₂; Hylmö

1955, figures 1 and 2; Brouwer 1956, figures 4 and 5). Kramer (1956 b) showed that in pea plants the active accumulation (B) of P^{32} was lowered by azide without effecting the transpiration component (D) of the ion absorption. Neither DNP nor NaN_3 affected the griseofulvin absorption connected with the transpiration, but at the same time the water independent absorption was very much reduced (Crowdy *et al.* 1956). An extensive material not yet published from this laboratory gives the same view of the influence of different inhibitors of the uptake of chloride in pea and wheat plants.

Methodics

With a micropotometer Brouwer determined at the same time the water flow in five 2.5 cm zones of unbranched roots of broad beans (*Vicia Faba*) at different suction tension from the shoot. The chloride uptake was determined as the loss from the medium. For details see Brouwer (1953, 1954, 1956).

Through the courtesy of Dr. Brouwer the material building the ground for figure 3 in Brouwer (1956) was handed over for further rearrangement. The writer is much obliged to Dr. Brouwer for an interesting personal discussion. It was hoped that this reinterpretation of the experiments could be published together with Dr Brouwer. Unfortunately this co-operation was not possible. Our points of view on account of the passive and active components in the ion transport proved after a long discussion to be too much divergent to make possible an article in our two names.

Pressure Difference and Water Flow

It has long been known that the water flow through the root with increased suction from the shoot increases to a greater extent than is to be expected from the change in pressure (Jost 1916, Romell 1918, Koehnlein 1930, Brewig 1937, Rosene 1941, Brouwer 1953, 1954, 1956). In the determinations by Brouwer on the roots of *Vicia Faba* the main part of the water with small differences in pressure goes into the apical zones. With increased pressure a still larger part of the water stream passes through the basal zones. The cause of this disproportion between pressure and water flow has been explained in different ways. Brewig (1937) and more recently Brouwer (1953, 1954, 1956) speak of a change, induced by the increased pressure, in the *resistance* or *conductivity* of the root to water flow. The changes in resistance or conductivity are thought to be controlled in any way by the living protoplasm probably by the turgescence of the tissue.

Hylmö (1955, figure 7) has reinterpreted the earlier experiment of Brouwer (1953, 1954) and has given the phenomenon the following interpretation, guided by the experimental background of the method worked out by Erbe (1933) for the determination of the pore size distribution of a membrane (cf. Bull 1943).

For the flow of liquids through a tube, where l is the length of the tube, r its radius, p the difference of pressure at the ends, η the coefficient of viscosity, the volume escaping per time is according to the Hagen-Poiseuille equation

$$V = \frac{p \pi r^4}{8 l \eta} \quad (1).$$

If we in the root zone have N such capillaries with an average pore radius of r_a , evidently the total volume of liquid, V_t , flowing through all the capillaries is

$$V_t = \frac{p \pi r_a^4 N}{8 l \eta} \quad (2).$$

Assuming that the form and number of the pores in the root do not change, the quotient $\frac{\pi r_a^4 N}{8 l}$ remains constant. If the temperature is unchanged, the viscosity of the liquid, η , (*i.e.*, of water in our case) also remains constant. On these presumptions the water flow in the root ought to be according to eq. (2) directly proportional to the difference in pressure, thus

$$V_t = K p \quad (3).$$

The equation formulated by Romell (1918), Sabinin (1925) and Brieger (1928) and later applied by Brewig (1937), Arisz *et al.* (1951), and Brouwer (1953, 1954),

$$V = K (S_{xyl} - S_{med}) \quad (4).$$

where $S_{xyl} - S_{med}$ is the pressure difference between the xylem and medium, is, as pointed out by Hylmö (1955), a conclusion of the Hagen-Poiseuille equation corresponding to eq. (3).

Hagen-Poiseuille's eq. (1) and (2) is strictly valid only for cylindrical pores. For flow through porous media Darcy's law is valid instead. Deducing this later law gives also the equations (3) and (4).

When liquid is pressed through very thin capillaries (ultrafilters), deviations from Hagen-Poiseuille's law are not infrequently found. With low differences in pressure a smaller volume of liquid passes through than what is required by direct proportionality, according to eq. (3).

According to eq. (2) the reduced water flow with low pressures can have three causes:

(a) the viscosity of the liquid, η , can increase on passage through narrow pores (compare Bingham's law and plastic flow);

(b) the "conductivity" can be diminished by a decrease in the pore size, r , or an increase in the pore length, l ;

(c) the number of pores in use, N , can be diminished, when at low pressures the liquid in the narrower pores not in use remains stationary.

Colloid chemists and not the least medical men prefer to speak of an

increased viscosity in liquids according to (a) in passage through capillaries under low pressure (cf. Frey-Wyssling 1937, Stålfelt 1956). Brouwer (1953, 1954) like Brewig (1937) earlier has chosen to speak of reduced conductivity of the root, which implies a displacement at low pressure in the quotient $\frac{I_n^4}{I}$, more precisely a decreased pore radius, r_a , as a result of changes in cellular turgor. Brewig (1937) has given a definition of resistance and conductivity which is in complete agreement with (b).

Hylmö (1955, figure 7) has shown that the pressure/water diagram of the root largely agrees with the one presented by Erbe (1933) in the determination of the pore-size distribution of ultrafilters, when water is forced through a membrane displacing air or isobutylic alcohol. An S-formed curve passes over into a straight line. Erbe (1933) gives the following interpretation of the flow-pressure curve. At a certain constant pressure on the filter the largest pores are opened for water. When the pressure is increased another group of smaller pores is opened, which is evidenced by a corresponding, rather than a proportional, increase in the flow through the filter (c). The discontinuous increase in the flow proceeds until all the pores are opened for water. With further increased pressure the increase in the water flow becomes proportional to the increase in pressure in accordance with Hagen-Poiseuille's law, eq. (2) and (3).

Erbe's (1933) interpretation is in our case with the root the most plausible of the three alternatives. *The pressure/water curve, which according to Cantor's law permits a determination of the pore size, gives a approximate value for r_a of 7—20 μ (Hylmö 1955). This value of the pore radius is of the same magnitude as for the interfibrillar capillaries in the cell wall determined by X-ray (Frey-Wyssling 1937) or by the rate of flow of gases as well as the electrical conductance through wood (Stamm 1946).*

As demonstrated by Frenzel (1929) particles of colloidal gold up to a diameter of 9 μ can diffuse through cell walls of *Pinus* free from pit-pairs. These determinations of the pore size agree fairly well with the new measurement of electronic microscope on similar objects (cf. Brauner 1956).

In Brouwer's main experiment a change in the size of the pores is not likely to be the case. The medium is kept unchanged during the course of the experiment. In order to facilitate the comparison with Brouwer's article the term "conductivity" will however be retained here for lack of a suitable term for the phenomenon (c), interpreted as a change in the number of pores in use. In other experimental series of Brouwer (1956) with inhibitors or with alteration of the composition of the medium, a change in the pore size, r , or the pore length, l , according to (b) can, of course, not be excluded. A combination of (b) and (c), a change in the pore size and the Erbe phenomenon at the same time, may give a plausible explanation of the results of all experiments.

According to Brewig (1937) and Brouwer (1953, 1954, 1956) the conductivity, C_{Br} , is equal to the reciprocal resistance, R_{Br} .

$$C_{Br} = \frac{1}{R_{Br}} \quad (5).$$

This implies that the constant, K , in eq. (3), according to Brewig is called conductivity. Even in deviations from Hagen-Poiseuille's law the following is thus valid:

$$V_t = C_{Br} p \quad (6).$$

It should be observed that when some older plant physiologists, for example Dixon (1914), speak of the occurrence of a greater resistance to flow, R_{Dix} , with a greater velocity of the current of water in the plant, they are thus speaking of an entirely different resistance. This resistance is simply the reciprocal value of the pressure loss from friction, p , which is expressed by Hagen-Poiseuille's equation (2), and thus has no connexion with the deviation from the direct proportional relation between pressure and water flow in eq. (2) and (3).

$$R_{Dix} = \frac{1}{p} \quad (7).$$

Recently Arisz (1956) and Brouwer (1956) have accepted if ever so hesitantly the deviation in the water flow as an *Erbe*-phenomenon. It is particularly valuable that Brouwer (1956, figure 12) has given new experimental results, now plotted in a pressure/water diagram. This new curve (Figure 1) agrees in form and position remarkably well with that constructed from Brouwer's earlier experiment (Hylmö 1955, figure 7). The collected material from all Brouwer's experiments (Brouwer 1956, figure 3) has also been recalculated and plotted in a pressure/water flow diagram, which in an elegant manner elucidates the conditions in the individual zones (Figure 3).

In experiments from this laboratory not yet published, carried out on roots of *Vicia Faba* with the micropotometer technics of Brouwer, typical *Erbe* curves were received (Kihlman).

Pore Size

Figure 1 shows the already mentioned relationship between pressure and water flow. This is an extremely interesting diagram. Three points at the lowest pressures lie beautifully in a line which can be extrapolated to the origin; two points at higher pressures on another line corresponding to a strongly increased water flow, and this line also points toward the origin. *According to Erde's interpretation the root has two separate passages for water; one passage with wider pores opened even at low suction tension; another passage is composed of narrower pores, into which the water first flows when the pressure is raised to 1.8 atms. or more. The pore size of the*

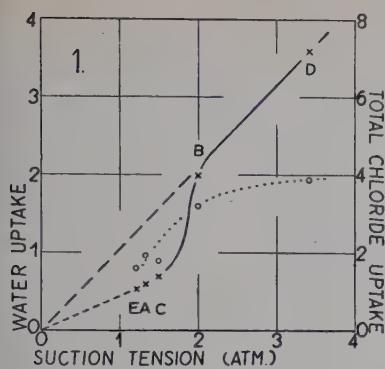


Figure 1. The water flow and the total chloride uptake at different suction tensions in zone III (5—7.5 cm from the tip) in the root of *Vicia Faba*.

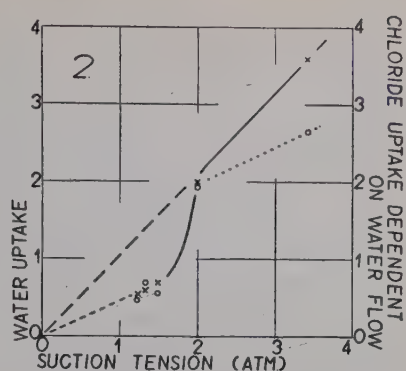


Figure 2. The water flow and the transpiration component of the chloride uptake at different suction tensions in zone III. The same experiment as in Figure 1. The concentration of the external medium was kept constant around the investigated root. The suction tension was varied by changing the concentration of the nutrient solution around the other roots. The water uptake is extrapolated to zero suction tension. Crosses denote water flow and circles chloride uptake. The letters A, B, C, D and E designate the chronological order of the determinations. Water uptake: 1 unit=200 mm³. water; chloride uptake: 1 unit=14 µg chloride both per 2.5 cm. root zone and 24 hours. From Brouwer (1956, figures 11 and 12).

wider pores cannot be read off from this diagram. On the other hand, according to Cantor's law the average radius of the narrower pores can be calculated to ca. 12 mµ (assuming that the pores are cylindrical). It is very interesting that the distribution of the pore size is extremely small (for method of calculation see Erbe 1933 and Bull 1943). All pores appear to have practically the same size. The smallest have a radius of ca. 10 mµ and the largest ca. 14 mµ. Earlier calculation according to the same method gave for *Vicia Faba* roots the average radius of ca. 7—20 mµ (Hylmö 1955).

It is especially interesting to compare the relationship pressure/water flow in the individual root zones in *Vicia Faba* (Figure 3). The water flow is determined by micropotometers simultaneously in five different 2.5 cm zones, numbered basally from the tip I, II, III, IV and V, on 16 occasions, both at low suction tension and at high. A noticeable deviation from Hagen-Poiseuille's law at low pressure differences appears in all zones with the exception of zone I.

At low pressure differences (below 1.8 atm.) the apical zones let through more water than the basal ones. The water flow diminishes regularly so that the farther from the tip the zone is taken, the less water passes through at the same pressure (Figure 3, at the bottom right). The difference in water

flow between the zones is so great that for these pores, which allow water passage even at low pressures, only an alteration in the pore length does not suffice as an explanation. According to eq. (2) the pore length must be increased five times in order to explain the difference in water flow between zone II (2.5—5 cm) and zone V (10—12.5 cm). The increase in the thickness of the root from the tip to zone V is only very insignificant. Instead the explanation probably lies in the fact that the pore radius decreases with increasing age. The difference in water transport between zone II and zone V corresponds according to eq. (2) to a decrease in the pore radius to two thirds if all pores still are in use. A closure of the transport paths with age to this degree does not seem at all unlikely. Some of the pores may close.

It remains to be ascertained which anatomic structure these wide pores, used for water transport even at low pressures, represent. These pores are so wide that within the investigated region of pressure down to 0.8 atm. no Erbe phenomenon occurred. The pressure/water curve seems to follow Hagen-Poiseuille's law. The average radius of these wide pores may be estimated to be greater than 25 μ even in zone V. It would be valuable, of course, to determine the relationship pressure/water also at very low pressure differences.

For the present we have to assume that these wider pores represent paths of water in the secondary cell walls not yet totally filled by cellulose fibrils. Between the groups of microfibrils there are wider channels than the normal intermicellar capillaries. In the zones IV and V the water flow at low pressure is only very low and we have to assume the cell walls to be nearly completed with the microfibrils tightly packed.

When the suction tension increases from 1.8 to 2.3 atm., the water flow in the zones II, III, IV, and V rises suddenly (Figure 3). As pointed out in the foregoing for the other material (Figure 2), the change lies within narrow pressure limits, indicating a very uniform pore size in the pores which are opened on the increased pressure. The pore radius can be estimated to ca. 10 μ (Figure 3). The pores of the basal zones are possibly somewhat narrower than those of the apical zones even in this pore group, which is first utilized for water transport at higher pressure differences.

At pressure differences of 2.3 atm. and above the water flow increases again in direct proportion to the rise in pressure in accordance with eq. (2) and (3). At the same pressure the zones III, IV, and V then let through the same amount of water; zone II, on the other hand, somewhat less and zone I considerably less. The question is wherein this difference between the zones lies. A possible explanation is that the actual pressure in zone I and in the apical part of zone II is not as high as in the other part of the root (Hylmö 1955). The suction tension has usually been determined in zone III. All the

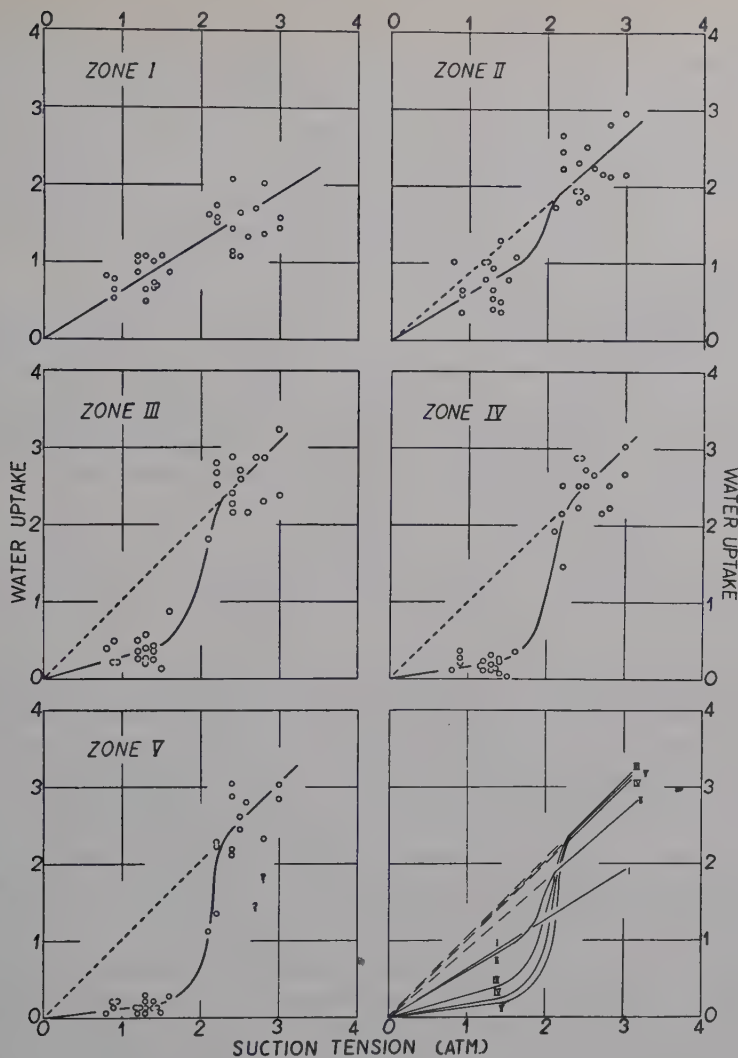


Figure 3. The relationship between suction tension and the water flow according to zones in the root of *Vicia Faba*. The water flow has been measured simultaneously in five zones in 16 parallel determinations at low and high suction tension. Each zone is 2.5 cm; zone I is the apical tip. At the bottom right the curves for the zones are compared. The values on which this figure is based have been earlier published by Brouwer (1956, figure 3).

Water uptake: 1 unit = 200 mm³ water per 2.5 cm. root zone and 24 hours.

suction tensions mentioned here are according to (2) pressure drops through friction, which we have to assume have arisen during the passage radially from the medium to the xylem, when the medium is flowing through cell walls, cytoplasm and vacuols.

Since the xylem in the apical part of the root is not fully developed, it can be expected that in the passage of relatively large amounts of water in the narrow transport paths a pressure loss through friction occurs even longitudinally in the apical zones. It is thus conceivable that with the water flow of 1.5 units in zone I (Figure 3) the pressure loss through friction longitudinally is 1 atm. and the actual pressure loss radially is not as in the basal zones 2.5 atm. but only 1.5 atm. Thereby it has been taken into account that the curves for all zones will coincide at full pressure.

On this assumption zone I has such wide pore size (radius $> 11 \text{ m}\mu$) that the Erbe phenomenon does not occur within the pressure region investigated ($< 2 \text{ atm.}$). In zone I the maximal pressure difference has not attained such a value that the finer pores are utilized for water transport. Differences in the anatomic structure apically and basally in the root are, however, so great that speculations on the nature of the pores are hazardous. The role of bleeding is also difficult to discern in this material.

The Nature of the Transpiration Induced Ion Transport

Brouwer (1953, 1954, 1956) found a strong connexion between the chloride uptake and the "conductivity" of the root. He believed that the "conductivity" of the root for water and for ions was changed parallelly, although without assuming a direct connexion between water flow and ion uptake. In his most recent investigation Brouwer (1956) demonstrated a direct connexion between the water "conductivity" of the root and the total chloride uptake of the plant (Figure 4). It will be shown here, however, that this connexion is only indirect.

An experiment with zone III (the piece 5—7.5 cm from the root tip), where the water flow and chloride uptake were determined in an elegant manner, has been accounted for in four diagrams from different aspects (Brouwer 1956, figures 10, 11, 12 and 13).

In Figure 1 the total chloride uptake is plotted. As Brouwer (1956) emphasizes, the connexion between water flow and ion transport does not seem to be particularly good plotted in this manner. However if one considers only the ion component dependent on the water flow, (D), the agreement is almost complete. When the accumulation (B) independent of the water flow, 1.25 units of chloride (each $14 \mu\text{g}$), is deducted (Brouwer 1956, figure 11), the water and chloride uptake coincide at the four lower pressures (Figure 2). On the other hand, at 3.41 atm. somewhat less chlorides are taken up than the direct proportionality between water and chloride requires.

Brouwer (1956) pays very great attention to the circumstance that in the experiment discussed the connexion between water flow and chloride uptake in zone III is not absolutely linear but somewhat curved (Brouwer 1956, figures 11 and 14). The accumulation is decreasing with the time. To be sure to have a real diversion from the straight line a more extensive material is needed. In the collected material of Brouwer, here given in the Figures 8 and 9, everything speaks for a rectilinear connexion in the separate zone. That the connexion between water and ion trans-

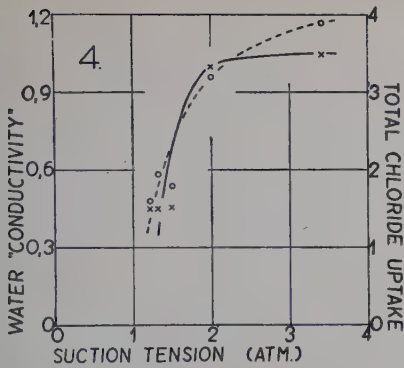


Figure 4. The water "conductivity" (full line) and the total chloride uptake (dotted line) versus suction tension. From Brouwer (1956, figure 13).

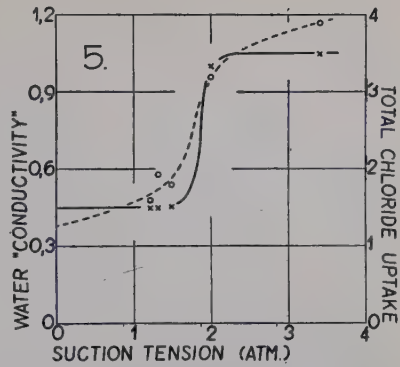


Figure 5. The water "conductivity" and the total chloride uptake versus suction tension. The curves are drawn in accordance with Figure 2 for "conductivity" and with Brouwer (1956, figure 11) for the chloride uptake. Crosses denote water "conductivity" and circles total chloride uptake. Water uptake: 1 unit = 200 mm³. water; chloride uptake: 1 unit = 14 µg chloride per 2.5 cm. root zone and 24 hours. Values from the same experiment as in Figures 1 and 2.

port in the entire root of *Vicia Faba* really is rectilinear is shown by all evidence in Brouwer's own material (Brouwer 1956, figure 19). This confirms the rectilinear connexion between water flow and ion or molecule transport observed by Schmidt (1936), Hylmö (1953), Crowdy *et al.* (1956), Koozin *et al.* (1956) and others.

Brouwer (1956) considered the agreement to be practically complete when he compared water "conductivity" with the chloride uptake, both plotted against the pressure (Figure 4). This agreement is, however, as will now be shown, only apparent and indirect. The lines in Figure 4, namely, are not drawn in accordance with that which can be discerned from the water and chloride uptake in the other diagrams. Instead the lines should be drawn as in Figure 5. Neither the "conductivity" nor chloride lines should point toward the pressure axis; instead they should cut the ordinates.

Figures 1 and 2 indicate that the value of the "conductivity" with pressure increasing from 0 to 1.5 atm. remains stationary at 0.45 and then rises abruptly between 1.5 and 2.0 atm. to a new constant value, namely 1.05. Of course we cannot be absolutely certain that in Figures 1 and 2 it is correct to extrapolate to origin. Under all conditions, however, the water/pressure line must cut the abscissa somewhere between 0 and 1.2 atm. It is hardly conceivable that the curve can fall more steeply than to 0.8 atm. The corresponding "conductivity" value for this extreme case is about 0.25. Whether in figure 5 the "conductivity" line runs toward 0.25 or, as dotted, toward 0.45 is of no consequence in our evaluation. The values for the root zones in figure 3 support the view that extrapolation to origin of the pressure/water curve is correct and thus Figure 4 gives a "conductivity" value of 0.45.

The chloride uptake has almost a rectilinear relationship with the water flow (Figure 6). Even without the passage of water chlorides are taken up (cf. Hylmö 1953), which implies that the chloride line should not point toward the pressure

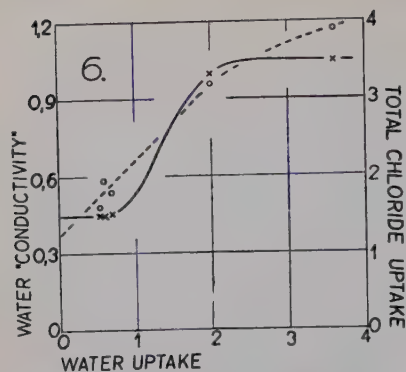


Figure 6. The water "conductivity" (unbroken line) and the total chlorid uptake (dotted line) versus water uptake. Crosses denote water "conductivity" and circles chloride uptake.

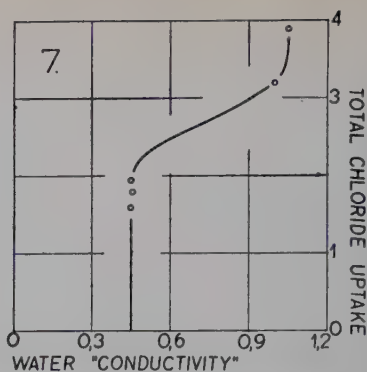


Figure 7. The relationship between water "conductivity" and the total chloride uptake. The values in the Figures 1, 2, 4, 5, 6, and 7 are taken from the same experiment, namely Brouwer (1956, figure 10, 11, 12 and 13).

axis as in figures 1 and 4 but should when extended cut the chloride axis as in figures 2 and 5. Even without the passage of water and at suction tension zero chlorides are taken up by the root.

From Figures 4 and 5 it appears that there is a certain similarity between "conductivity" and the chloride uptake plotted against the pressure. If, on the other hand, as in Figure 6 the "conductivity" and chloride uptake are plotted against the water flow, every similarity disappears. "Conductivity" plotted against water flow still has a marked S-form, although somewhat less pronounced than in the pressure diagram (Figure 5). *The chloride line, on the other hand, curves only slightly without any suggestion at all of an S-form. This shows clearly that the relationship between water flow and chloride uptake is direct. The chloride uptake increases proportionally with the water flow. Only by virtue of the fact that both the water flow and the "conductivity" are functions of the pressure, eq. (6), is there a connexion between "conductivity" and chloride uptake, but this relation is indirect.*

That any direct connexion does not exist is perhaps most evident in a direct comparison between "conductivity" and the chloride transport. The connexion water "conductivity"/chloride uptake has an S-form. The chloride uptake rises from 0 to 2.0 units while the "conductivity" remains stationary at 0.45 (figure 7). Whereas the chloride uptake later increase from 2.0 to 3.2 units, the "conductivity" increases abruptly from 0.45 to 1.05 and remains stationary there while the chloride increases up to 3.9 units.

The conditions are the same for the other experimental material. In Figure 3

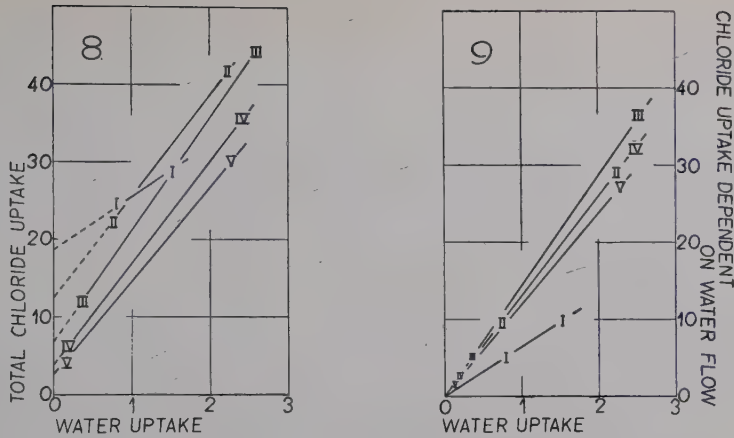


Figure 8. *The relationship between water flow and total chloride uptake. Roman numerals designate the root zones counted from the tip. Water uptake: 1 unit = 200 mm³, water per 2.5 cm root zone and 24 hours. Chloride uptake: 1 unit = 1 μ g Cl per 2.5 cm root zone and 24 hours.*

Figure 9. *The relationship between water flow and the transpiration component in the chloride uptake. The values are taken from Figure 8.*

is shown the relationship between suction tension and water flow for the five individual zones. The data are based on 16 parallel determinations at low and high pressure differences. Contemporary data on the chloride uptake, also taken from the original material on which Brouwer's figure 3 (1956) is based, were obtained from 12 parallel determinations carried out simultaneously in each of the five zones, partly at low and partly at high pressure differences. The mean values of this chloride uptake can be compared with the mean values for the water flow (Figures 8 and 9).

In Figure 8 the total chloride uptake has been divided by means of extrapolation into one component independent of the water flow, conceived as the diffusion component (A) together with the active accumulation (B) corresponding to the chloride uptake without water flow, and another component connected with the water flow (D). According to the methodic the diffusion component (A) here is negligible. The accumulation (B) is greatest in the apical zone and falls basally. It is thus in zones I to V: 19.0, 12.5, 6.8, 3.8 and 2.8 μ g. chloride per 2.5 cm root zone and 24 hours. The basally falling rate of chloride accumulation agrees with the earlier results with *Vicia Faba* (Hylmö 1955; Brouwer 1956, figure 8) and with the rate of accumulation of phosphate (Brouwer 1956, figure 9) and P³² in roots of barley seedlings (Kramer 1956 b).

If we consider only the water-linked chloride uptake (D), which our dis-

cussion primarily concerns we find that at the same rate of water flow the ion transport is practically equal in the zones II, III, IV and V (Figure 9). It is particularly interesting that even at low suction tension the chloride uptake in the different zones lies rectilinearly with the water flow. This occurs in spite of the fact that the suction tension and the "conductivity" varies widely (cf. Figure 3). As in the individual zones (Figures 6 and 7), the chloride uptake on comparison between the zones is directly linked to the water flow but only indirectly to the pressure difference and "conductivity".

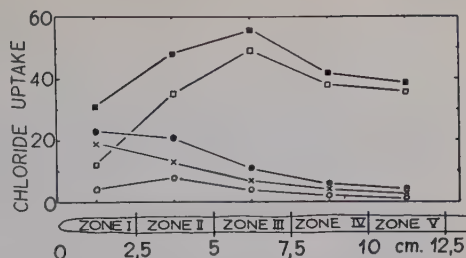
Specially interesting is the fact that the solution fluxing into the root at low differences in suction tension, when only the wider pores are in use for the transport, has the same chloride concentration as the flux of solution when also the narrow pores are in use at high pressure. In both cases the medium is diluted to ca 18 per cent when passing into the root. Figure 9 is based partly on the same material as earlier reported (Brouwer 1956, figure 8) where the influx coefficients are reported to be from top to base 0.054, 0.074, 0.13, 0.13 and 0.13. In Figures 8 and 9 the influx coefficients are now somewhat higher. From the tip to the base they are according to zones 0.09, 0.18, 0.20, 0.18 and 0.17. Zone I has thus a considerably lower coefficient than the other zones.

Figure 10 gives a résumé of the components of the zonal ion uptake at low and at high transpiration. The findings are in accordance with the figures of Wiebe and Kramer (1954) for the zonal uptake of P^{32} in barley roots.

The fixed influx coefficient in the zones II to V at low and at high pressure differences prove that chloride ions are passing through the root without being involved in the metabolism. It is difficult to see how, in all situations in younger as well as in older parts of the root, at low and at high water flux and at all suction tensions and conductivities an active mechanism should be able to transport ions through the root in direct proportion to the water flow. An active mechanism ought to give a high concentration at low water flow and a low concentration at rapid flux. Figures 3, 8, and 10 indicate that still older parts of the root than zone V will, at high pressure differences, let through water and ions in about the same extension as now shown for the zones IV and V. As Hylmö (1953) and Kylin and Hylmö (1957) have demonstrated for the ion uptake of the whole plant and Crowdy *et al.* (1956) for antibiotics, the same dilution occurs at different concentrations. Active co-operation in this transport seems very unlikely.

According to Hylmö (1953) the constancy of the influx coefficient is due to water having two pathways, but the ions only one, when drawn in mass flow through the root. The fraction of the medium solution which is drawn to the anticlinal cell walls of the epidermis is passing without any hindrance.

Figure 10. The zonal chloride uptake at low and high rate of transpiration in the root of *Vicia Faba*; active and passive components. Chloride uptake μg per 24 hours per 2.5 cm. root zone. Crosses denote active accumulation (B); circles low rate of transpiration (1 atm. suction tension); squares denote high rate of transpiration (3 atm. suction tension). Open circles and squares: transpiration component of chloride uptake (D), filled circles and squares total chlorid uptake.



In this part of the cell water as well as ions can freely pass. On the other hand the flux meeting the vacuol of the epidermis is filtered. Water is passing the tonoplast but not the ions. The ions are stopped at the tonoplast and will by diffusion partly return to the medium.

The influx coefficient is in this way another expression of the free space. It is hoped in a later publication to give more attention to this opinion. Here only a working hypothesis will be forwarded that the lower influx coefficient of zone I is due to the fact that in the youngest zone of the root the cell walls are a lesser part of the cell than in the older part of the root.

Conclusion

The analysis of Brouwer's (1956) experiments thus shows that his hypothesis (Brouwer 1953, 1954, 1956), "that the increased ion uptake, as it is found at a higher transpiration, is to be attributed to an increased conductivity for ions in the root tissue", lacks experimental bases. On the other hand, there appears to be a direct relationship between water flow and ion uptake. Future investigations will show how great a part of the water-linked ion component is a passive mass movement of the solution through the root without involvement of the transported ions in the root metabolism, and which part the water flow has in merely further transporting the ions earlier accumulated through the bleeding mechanism. The material now accounted for indicates that mass flow in the root of *Vicia Faba* is the completely dominating component. This is in full agreement with earlier findings in other plants for different kinds of ions (Hylmö 1953, 1955, Epstein 1956b, Kramer 1956 b, Kylin and Hylmö 1957).

In a publication which arrived after the completing of this paper Mees and Weatherley (1957) have demonstrated that in the roots of tomato plants the diffusion of water across the cortex was greatly facilitated by a pressure gradient. The fact that raising the pressure on the external medium also causes a reduction in diffusion resistance, in the opinion of the writers, does not support the turgidity hypothesis of Brouwer (1954).

Summary

Hagen-Poiseuille's law is valid for the water passage through the root. Deviations from the law occur at low pressure differences. Then less water flows through the root than is to be expected with the direct proportionality between pressure and water flow. In the root tip, however, no deviation was observed.

The deviation is considered to be an Erbe phenomenon, in that at low pressure differences the water remains stationary in the narrower pores. Increased viscosity of the liquid, decreased pore size or increased pore length, on the other hand, are not regarded as the cause of the reduction in the water flow.

Two types of pores are distinguished. A wider pore group lets through water even at low pressure differences. These pores grow narrower or fewer with increasing age of the root zone. In another, small sized pore group the water flows first at higher pressure.

The deviation from Hagen-Poiseuille's law makes it possible to determine the average radius of the narrow pores to ca. 10—12 m μ . These pores are assumed to represent the capillaries between the microfibrils in the cellwall.

Two components in the chloride absorption through root zones are distinguished. The active accumulation to the root itself is greatest in the root tip and decreases basally.

The transpiration component in the chloride transport is shown to be passive and directly linked to the water flow and only indirectly to the pressure difference and the "conductivity". The ions of the true transpiration stream are passaged through the root without being involved in the metabolism of the root.

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Pigment Formation and Growth in Blue-Green Algae in Crossed Gradients of Light Intensity and Temperature

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The blue-green algae contain chlorophyll *a*, phycocyanin, phycoerythrin, and a number of yellow pigments, mainly carotenoids. The color of the blue-green algae may vary greatly within the same species when grown under different conditions. These color variations are due to changes in the pigment ratios in the algae. Light is supposed to be a factor affecting these changes; both its intensity and its spectral composition have been assumed to be important in this respect. Oltmanns (1892) showed that the blue-green algae assumed different colors when grown at high and low light intensity, and Engelmann and Gaidukov (1902) reported that the color of some blue-green algae depended upon the spectral composition of the light, and that the resulting color was complementary to that of the light to which they were illuminated. Since that time a number of observations have been gathered on the effect of light on the color of algae. Two theories, founded upon entirely different principles arose, namely: (1) the color that the algae assume is a *complementary chromatic adaptation* depending upon the spectral composition of the light; *e.g.*, in the sea the red algae at greater depth have a clear red color which is complementary to the blue-green light extending to this depth; (2) the algae at low light intensity will form more accessory pigments effective in photosynthesis so they catch a high percentage of the

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light; *e.g.*, in the sea at greater depth, the red algae are shadow plants with a high amount of accessory pigments effective in photosynthesis (*light intensity adaptation*). At present there is no general agreement as to which of the two theories above is right (for review of the literature on this subject see Rabinowitch, 1945 p. 424).

Factors other than the light intensity most certainly are also involved in the processes affecting the pigment ratios in plants. The temperature under which they are grown is one.

Recently an apparatus has been built in which algae, grown on agar, are exposed simultaneously to crossed gradients of light intensity and temperature (Halldal and French, 1956). This apparatus gives the opportunity for study of the growth of the organism and of the formation and destruction of pigments under different light intensity and temperature conditions, and also for direct study of the interaction of these two factors.

Anacystis nidulans Drouet

When the blue-green alga *Anacystis nidulans* Drouet (Kratz and Allen's strain; see Kratz and Myers, 1955) was grown in the apparatus, there was a striking difference in color in different parts of the growth area. Figure 1 shows a series of photographs taken at 12 hour intervals during an experiment. At the same time a record of the changes in appearance was made with color film.

After 12 hours above 45°C at 1,000 foot-candles and above 50°C at 25 foot-candles, the alga was killed and completely bleached. It had grown significantly from 33 to 44°C at 1,000 foot-candles, and from 28 to 45°C at 25 foot-candles. The color within these limits was fresh green. At lower temperatures at all intensities the inoculum showed little change, if any.

After 24 hours a striking difference in the color in different parts of the growth area started to develop. The alga was now completely killed at temperatures above 44°C at 1,000 foot-candles, and above 45°C at 25 foot-candles. At high temperature close to the killing boundary, and at light intensities from 400 to 1,000 foot-candles, the color was bluish-green, presumably owing to greater phycocyanin formation under these conditions. The blue-green color was most striking from 500 to 800 foot-candles. At light intensities from 500 to 1,000 foot-candles, and at temperatures from 30 to 42°C, the color was changed from green to yellow, mainly due to destruction of phycocyanin (see p. 412). At light intensities from 25 to 500 foot-candles, and at temperatures between 25 and 45°C the color was somewhat deeper green, presumably due to high chlorophyll content. At lower temperatures and light intensities, from 13 to 25°C, and from 25 to 400 foot-candles, the

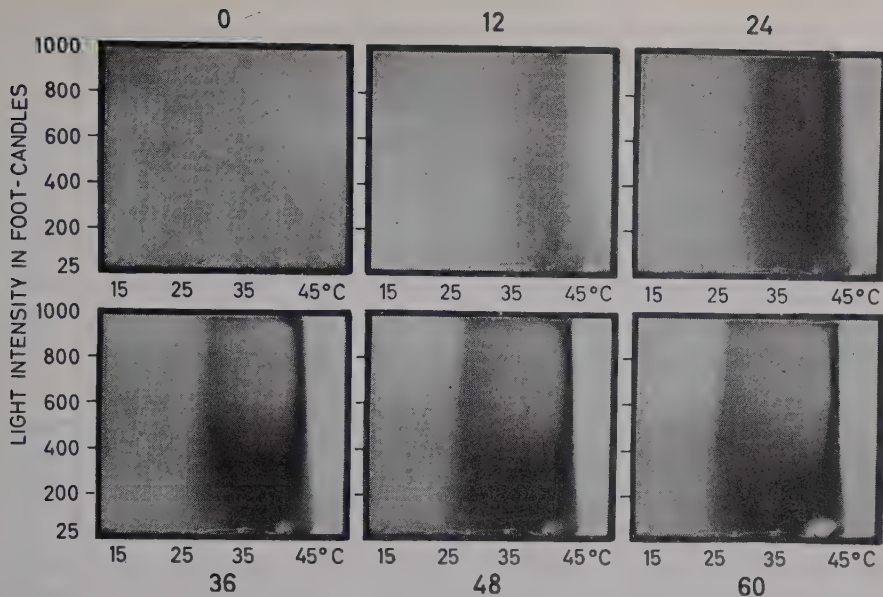


Figure 1. Photographs taken at 12 hours intervals showing the growth pattern of *Anacystis nidulans* when exposed to crossed gradients of light intensity and temperature. The figures at each picture show numbers of hours after the gradients were set up. For color description see text.

inoculum remained unchanged, while the alga at these same low temperatures, but at higher light intensities, started to bleach out in 24 hours.

During the rest of the experiment this pattern showed only small changes, but the boundaries between the different zones developed more sharply, and the zones themselves became more strikingly different. However, one of the boundaries in particular was not stationary; namely the one at 25 to 32°C parallel to the light intensity scale. This boundary moved slowly during the experiment toward lower temperatures. After 24 hours it was found at 32°C, and, after 60 hours, at the end of the experiment it was at 25°C. Several experiments with this alga show that this is the pattern that develops under normal conditions. However, the amount of inoculum will change the time for development. If a very light inoculum is used, the pattern of 12 hours in the above description will not occur. On the other hand, a heavy inoculum will keep the condition of 12 hours in Figure 1 for some time, from one to three days, but the final pattern under normal conditions will be like that described above.

In order to analyze the pigments formed under different conditions, pieces of agar with the alga were cut off the plate and placed upon a piece of opal glass. The absorption of the samples was measured in a Beckman DK-2

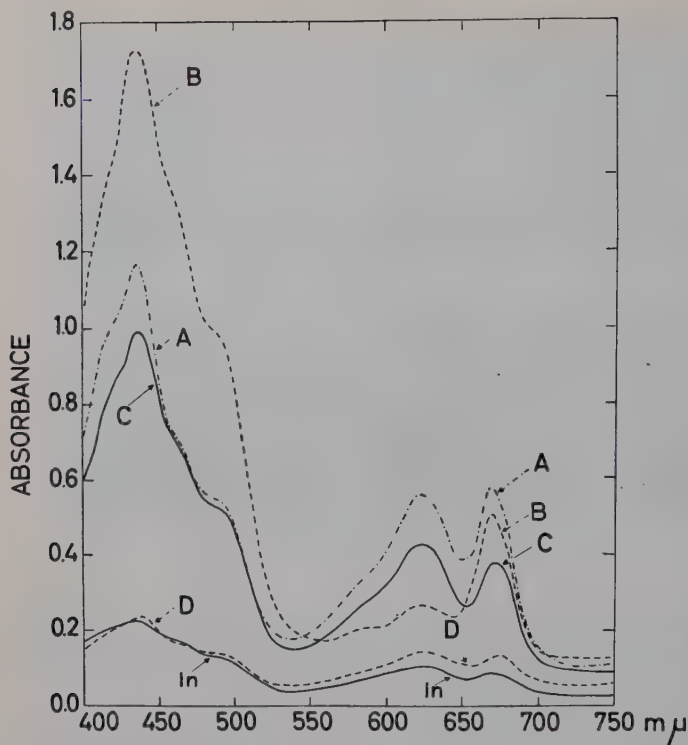


Figure 2. Absorption curves of the inoculum (In), and of four samples at the end of an experiment showing variations in the relative and absolute pigmentation of different samples. A: sample from 30°C, 100 f.c.; B: 38°C, 900 f.c.; C: 45°C, 250 f.c.; D: 15°C, 100 f.c.

spectrophotometer. A piece of agar not containing the alga, also attached to opal glass was used as a blank. This procedure is based upon the diffusing plate method of Shibata *et al.* (1954) and was suggested for this purpose by Shibata (pers. comm.). In the experiment that is described below, a rather heavy inoculum was used. The samples were taken after two days of growth, but the pattern was somewhat similar to that for 24 hours in Figure 1.

In Figure 2 are shown absorption curves of the inoculum used and of four samples taken from different parts of the growth area. Phycocyanin has an absorption maximum around 620 $m\mu$, and the red absorption maximum of chlorophyll-*a* *in vivo* is around 670 to 680 $m\mu$. The relative heights of the absorption curves at these wavelengths will thus give information about the relative amount of phycocyanin and chlorophyll *a* present in the sample. It is evident that the relative amount of phycocyanin and chlorophyll *a* varies greatly with growth conditions. There is also a significant difference in the shape as well as in the wavelength position of the red absorption peak of chlorophyll *a*. Furthermore the amount of yellow pigments formed at different parts of the growth area was highly variable, as is shown by the change

in shape and height of the curves in the blue part of the spectrum. Also in this part of the spectrum a slight wavelength shift of the maximum is indicated.

By using the curve analyzer of French *et al.* (1954) a quantitative analysis of the observed spectral differences has been attempted. In the first place the *in vivo* absorption characteristics of the principle individual pigments present in *Anacystis* can be estimated. From these characteristics it is then possible to estimate the quantitative relations between the various pigments in the original samples, and thus to obtain pertinent information on the interaction of light and temperature in pigment synthesis.

Phycocyanin

As mentioned above, phycocyanin has an absorption maximum in solution around 620 m μ (Svedberg and Katsurai, 1929). In order to derive the phycocyanin absorption curve for the intact pigment in *Anacystis*, two absorption spectra were chosen which were very similar in shape except for the spectral region from 550 to 660 m μ , where differences were evidently caused by different phycocyanin contents. The absorption spectra of two samples, one taken from 37°C and 600 foot-candles, and the other from 33°C and 600 foot-candles, had these characteristics (Figure 3). The curves were adjusted for equal chlorophyll content by making them coincide in height at the chlorophyll *a* red absorption peak at 670 m μ , and the 33°, 600 f.c. curve was then subtracted from the 37°, 600 f.c. curve. The resulting difference curve had a maximum at 625 m μ , and a shape very similar to the phycocyanin absorption spectrum of Svedberg and Katsurai (*l.c.*), and also to the curve obtained by Latimer (1956) for phycocyanin. There is, however, a significant difference in the peak position. Svedberg and Katsurai found the maximum at 616 m μ , and Latimer at 620 m μ . The position of the phycocyanin peak may, however, be different in solution and in living algae, and different treatments during the extraction may also possibly influence the wavelength position of the peak. When different samples were used in our analysis, the resulting phycocyanin absorption curves revealed some variations, both in peak position and in shape. The peak position in our analysis is expected to shift if absorption curves of some of the carotenoids have long "tails" extending into this part of the spectrum, and the amount of these carotenoids varies. The differences might also possibly be caused by minor pigments absorbing around 500 to 650 m μ ; *e.g.*, phycoerythrin. The presence of phycoerythrin, which some blue-green algae contain in small quantities, was, however, not revealed in the absorption spectra of *Anacystis*.

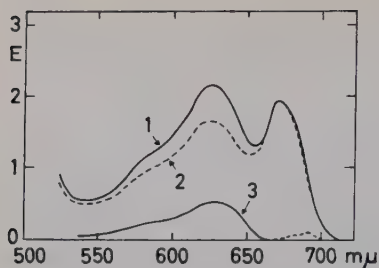


Figure 3. The deviation of the phycocyanin (3) absorption curve. (1) sample from 37°C, 600 f.c.; (2) 33°C, 600 f.c. E=Absorbance (Arbitrary units).

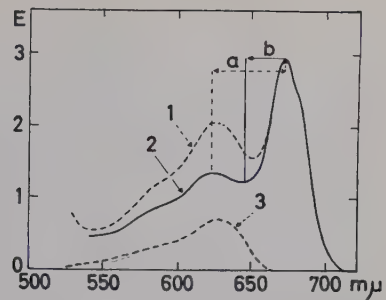
The difference curve of Figure 3 has been chosen as our phycocyanin absorption curve because it was derived from absorption spectra of *Anacystis* which at wavelengths other than from 550 to 600 $m\mu$ had the greatest similarity; and because it had a great similarity to the absorption curve of phycocyanin in solution, except for its peak position.

Chlorophyll a

Chlorophyll *a* is the only known chlorophyll in blue-green algae. *Anacystis* therefore gave a good opportunity to study the spectrum of this pigment *in vivo*. Above 550 $m\mu$, the absorption of *Anacystis* is predominantly caused by two pigments: chlorophyll *a* and phycocyanin. Subtracting the right amount of phycocyanin from an absorption spectrum of *Anacystis* should give the absorption of chlorophyll *a* in this region of the spectrum. In order to decide on the proper amount of phycocyanin to subtract, certain assumptions had to be made.

From absorption spectra of other plants containing no chlorophyll *b*, and such small amounts of other chlorophylls that their presence was not revealed in the absorption spectra of the living plants, it was deduced that the distance between the red absorption peak of chlorophyll *a* and the nearest valley at shorter wavelength is 28 $m\mu$. From the red absorption peak to the next maximum at shorter wavelength the distance is 48 $m\mu$. These figures came from Shibata (1958) for *Neottia*, and Halldal (1958) for *Dinophyceae*. About the same distances are found in organic solvents also; 26 and 46 $m\mu$ respectively (for reviews see Rabinowitch, 1951, and Smith and Benitez, 1955). If the phycocyanin curve of Figure 3 is subtracted from the absorption spectrum of *Anacystis*, the distances from the red absorption peak in the difference curve to the nearest valley and the nearest peak at shorter wavelength will vary with the assumed relative proportion of the two pigments. In constructing Figure 4, this proportion was chosen such as to give the above mentioned numerical values of these two spectral distances.

Figure 4. The deviation of the combined chlorophyll *a* (2) absorption curve in the red part of the spectrum. Phycocyanin (3) from Figure 3. (1) sample from 31°C, 600 f.c. a=48 mμ; b=28 mμ.



Chlorophyll a, 670 mμ, and *Chlorophyll a*, 682 mμ

When absorption curves of samples originating from different parts of the growth area were compared, it was evident that the position of the red absorption peak shifted (see Figure 2). In most cases it was at 670 mμ, but at temperatures around 24 to 25°C, it was at 675 mμ. Corresponding to this shift, there was also a small change in the wavelength position of the blue absorption peak from 435 mμ to slightly below 440 mμ. Another feature of the curves is a shoulder around 680 mμ. This shoulder was more or less pronounced in various samples. It was most clearly visible in samples from very low temperature and at extremely high temperatures close to the killing boundary. Very low light intensity also made this shoulder show up. This suggested that two components contribute to the red absorption peak of chlorophyll *a*.

In Figure 5, an absorption curve from a sample taken from 37°C and 900 foot-candles is subtracted from one taken at 24°C and 250 foot-candles. The resulting difference has a great resemblance to the absorption curve of chlorophyll *a*, with a maximum at 682 mμ. It was therefore assumed that two different forms of chlorophyll *a* contributed to the absorption by *Anacystis*; one with maximum absorption in red light at 670 mμ, and the other with maximum absorption at 682 mμ. Variations in the relative amounts of these two components explain the change in peak position and in the shape of the curves.

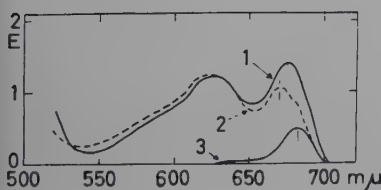


Figure 5. The deviation of the chlorophyll *a* 682 mμ (3) absorption curve in the red part of the spectrum. (1) sample from 24°C, 250 f.c.; (2) 37°C, 900 f.c.

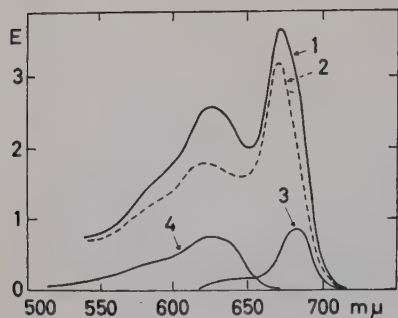


Figure 6. The deviation of the chlorophyll *a* 670 mμ (2) absorption curve in the red part of the spectrum. Phycocyanin (4) from Figure 3. Chlorophyll *a* 682 (3) from Figure 5. (1) Sample from 31°C, 600 f.c.

Figure 6 shows how the spectrum of chlorophyll *a*, 670 mμ was derived from the absorption spectrum of the sample illustrated in Figure 4 by subtraction of (1) the phycocyanin spectrum (Figure 3), and (2) the spectrum of chlorophyll *a*, 682 mμ (Figure 5), in such relative proportions that the shape of the difference curve at wavelengths longer than 670 mμ is similar to that of chlorophyll *a* in solution at wavelengths longer than its red absorption maximum.

The phycocyanin curve of Figure 3, the chlorophyll *a*, 670 mμ curve, and a curve similar in shape to the chlorophyll *a*, 670 mμ curve, but with a maximum at 682 mμ (chlorophyll *a*, 682 mμ), were all traced and added together in the curve analyzer of French *et al.* (1954). It was then possible, by choosing the proper values of the "absorbance" scales of these curves, to reproduce the absorption curve from 600 mμ to longer wavelengths of any sample of *Anacystis*. The fit was closer than 10 per cent.

Yellow Pigments

The absorption of *Anacystis* in the blue part of the spectrum is caused by a number of different pigments. Chlorophyll *a* has a high absorption in this region, and the carotenoids absorb mainly at these wavelengths. There are other yellow pigments present in the algae also. For example, Forrest *et al.* (1957) have analyzed the formation of pteridines in *Anacystis*, and they have shown that their formation was intimately concerned with the photosynthetic activity of the alga.

From curve analysis it was evident that the relative amount of the different yellow pigments varied over a wide range. It was therefore not possible to derive a single curve for the sum of these pigments. However, as some of the curves have a very similar shape, but a different magnitude in this region, their main difference must be of a quantitative nature. By the use of such curves it should be possible to derive the absorption spectrum of chlorophyll *a* in the blue region.

Blue Absorption Spectrum of Chlorophyll *a*

The absorption spectrum of a sample at 27°C and 900 foot-candles, and one from 33°C and 600 foot-candles were very similar in shape in the blue and green region of the spectrum. In order to make the chlorophyll content of these equal, they were made to coincide at their red absorption peak, and the curve of 33°C, 600 foot-candles was subtracted from that of 27°C, 900 foot-candles, thus giving a curve representing the yellow pigments in these two samples (Figure 7). This curve was then used to subtract the yellow pigments from the curve of sample 33°C, 600 foot-candles. Some assumptions had to be made in deciding on the appropriate factor to be applied to the curve before subtraction.

It is known from solutions that the ratio blue absorption peak/red absorption peak of chlorophyll *a* is about 1.30, somewhat depending upon the solution (from 1.25 to 1.40); see Rabinowitch (1951) and Smith and Benitez (1955). It was assumed that the ratio between these two chlorophyll *a* peaks *in vivo* was about the same. Further, it is known that the absorption of chlorophyll *a* in solution is very low, practically zero, around 475 m μ . It was therefore assumed that chlorophyll-*a in vivo* has a very low absorption somewhere between 475 and 500 m μ . The absorption factor for the yellow pigment curve was then computed to meet these requirements (Figure 8).

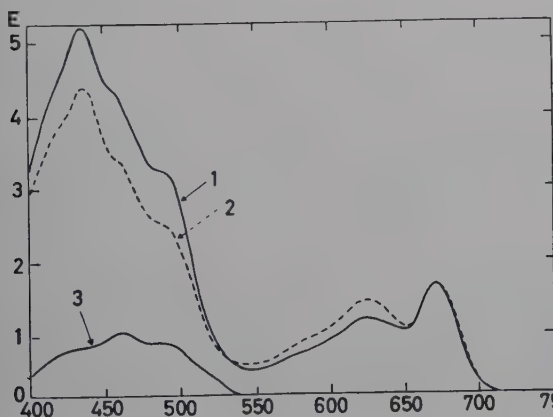


Fig. 7.

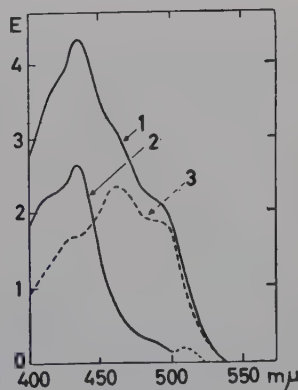


Fig. 8.

Figure 7. The deviation of the absorption curve representing the yellow pigments (3) in samples taken from 27°C, 900 f.c. (1) and from 35°C, 600 f.c. (2).

Figure 8. The deviation of the combined chlorophyll *a* (2) absorption curve in the blue part of the spectrum. (1) sample from 35°C, 600 f.c. Yellow pigments (3) from Figure 7.

The resulting curve is very similar in shape to the absorption curve of chlorophyll *a* in the blue part of the spectrum.

It was further assumed that the blue absorption of chlorophyll-*a* *in vivo* is caused by two different components, as is the case in the red region of the spectrum. Since the chlorophyll *a* of the sample 33°C, 600 foot-candles was found to consist mainly of the 670 mμ form, it was assumed that the blue absorption peak of chlorophyll *a*, 670 mμ is where the difference curve of Figure 8 has a maximum, namely at 435 mμ.

The slight shift in the blue absorption peak mentioned on page 407 is the only indication of the presence of two chlorophyll *a* forms in the blue part of the spectrum. The difference between these two forms in the red part of the spectrum was found to be 12 mμ. As the difference in the peak position at different wavelengths is on a frequency basis, the peaks would be only 8 mμ apart at wavelengths around 440 mμ. Due to the many substances absorbing in the blue part of the spectrum, a clear distinction between two peaks in such a position would be very difficult to observe.

The Final Curves in the Visible Region

Based upon the above analysis, the curves of Figure 9 were drawn. At wavelengths longer than 600 mμ, it has been possible to reproduce any absorption spectrum from *Anacystis*, by adding the appropriate amount of each of the curves in Figure 9. In our analysis there has been only one minor detail indicating the presence of two chlorophyll *a* forms in the blue region of the spectrum, and our analysis did not reveal the small bands in the absorption curves of chlorophyll *a* around 500 to 600 mμ, which are known from solutions. In order to indicate these uncertainties, the absorption curves of chlorophyll *a* have been dotted at wavelengths shorter than 600 mμ.

Phycocyanin has a slight increase in absorption from 500 mμ toward shorter wavelengths in solution (Svedberg and Katsurai, 1929; Latimer, 1956). The shape of the phycocyanin curve from 525 mμ to shorter wavelengths is based upon this.

Figure 10 shows as an example how the absorption spectrum of a sample from 37°C and 600 foot-candles can be decomposed into a number of individual pigment spectra. The curve marked yellow pigments is here derived from the original absorption curve by subtraction of the two chlorophyll curves and the phycocyanin curve.

Pigments Formed at Different Locations in the Growth Area

As any curve could be separated into its main components: chlorophyll *a*, 670; chlorophyll *a*, 682; phycocyanin; and yellow pigments; this gave oppor-

Figure 9. The absorption curves of: phycocyanin (3), chlorophyll *a* 670 $m\mu$ (1), and chlorophyll *a* 682 $m\mu$ (2). The uncertainty of a few assumptions in the yellow to violet region of the spectrum has been indicated by making the lines of the chlorophyll *a* curves dotted.

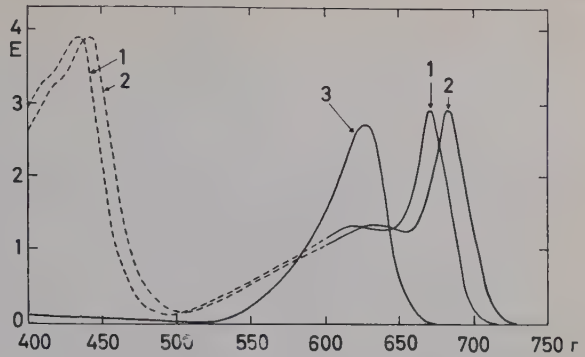
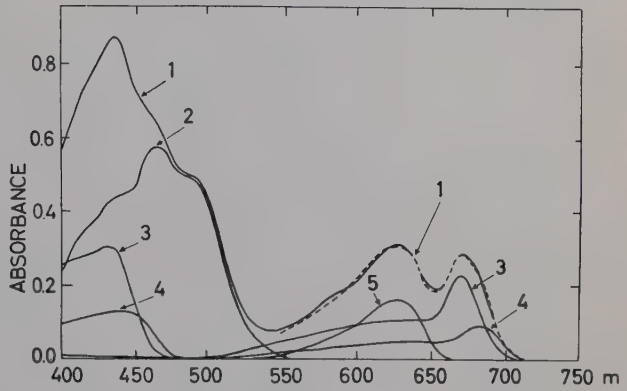


Figure 10. The absorption curve from a sample grown at 37°C. 600 f.c. (1) separated into its phycocyanin (5), chlorophyll *a* 670 $m\mu$ (3), chlorophyll *a* 682 $m\mu$ (4), and yellow pigment components (2). The dotted line is the sum of chlorophylls and phycocyanin.



tunities for the study of the formation of these different pigments at different temperatures and light intensities. The values necessary for this study can be obtained by calculations from simple equations based upon the absorbance at 625 $m\mu$ (phycocyanin); 670 $m\mu$ (chlorophyll *a*, 670); 682 $m\mu$ (chlorophyll *a*, 682); and 465 $m\mu$ (yellow pigments) more easily than by subtracting curves.

Figure 11 shows a three-dimensional representation of the relative amount of phycocyanin; chlorophyll *a*; and yellow pigments at different places in the growth area. This represents the growth pattern before the yellow region in the center started to develop, in this experiment 48 hours (cp. Fig. 1, 24 hours). A few irregularities in the pattern were corrected by interpolation.

The chlorophyll content was highest around 300 foot-candles between 30 and 40°C, but it was also high over a rather wide temperature range around this light intensity, and over a wide light intensity around 30°C.

For phycocyanin the formation was highest at high light intensity and temperature, with a decrease toward extreme light intensity, and a very sharp

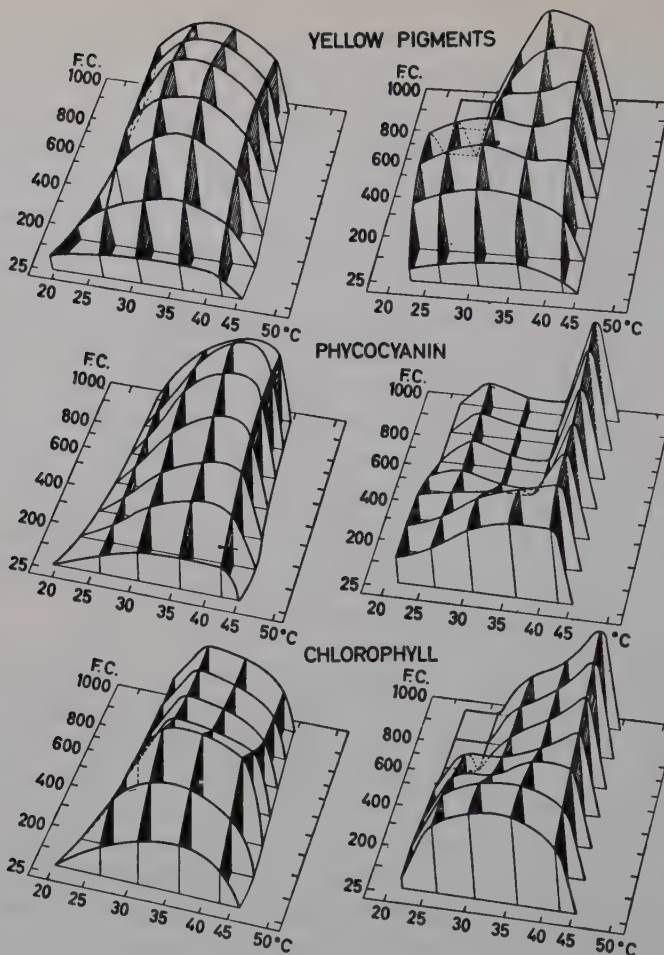


Fig. 11.

Fig. 12.

Figure 11. *Three-dimensional representation of total chlorophyll a, phycocyanin, and yellow pigments at an intermediate stage (48 hours in this experiment) in the development of the growth pattern.*

Figure 12. *Three-dimensional representation of total chlorophyll a, phycocyanin, and yellow pigments after the final growth pattern was developed (60 hours in this experiment).*

drop at extreme temperature. The phycocyanin content lessened both toward lower light intensity and temperature.

The formation of yellow pigments was greatest between 400 and 800 foot-candles, and from 30 to 35°C. Thus the formation of yellow pigments had a maximum between those of chlorophyll and phycocyanin.

The situation described above was not stationary, but an intermediate stage

leading into the striking pattern illustrated in the latter part of the experiment of Figure 1. A three-dimensional representation of the pigment composition within the pattern of Figure 1, 48 hours, is given in Figure 12 (in the present experiment this pattern was actually attained after 60 hours).

The chlorophyll content was highest at the highest temperature and light intensity within the growth area, but a high chlorophyll content was found at all light intensities, and increasing with increasing light intensity, within the narrow stripe from 43 to 45°C. Also at low light intensity, around 100 foot-candles, over a wide range of temperature from 26 to 45°C, the chlorophyll content was very high with a maximum at 35°C. Both from the narrow high temperature stripe and the wider area in the low intensity region, the content decreased evenly toward the middle of the growth area. A very sharp drop was found at 26°C at all light intensities.

The narrow stripe at high temperature was set apart from the central part of the growth area mainly by its high phycocyanin content, which lessened rapidly with decreasing temperature, and which also decreased slightly with decreasing light intensity within this narrow stripe. A rather high phycocyanin content was also recorded at low light intensity over a wide range of temperature from 26 to 45°C. Both from the narrow high temperature stripe and the wider area in the low intensity region the phycocyanin content decreased rapidly toward the middle of the growth area where a very low phycocyanin content was recorded. At light intensities above 400 foot-candles there was a slight increase in phycocyanin content from the central area toward lower temperature with a maximum at 26°C followed by a rapid decrease.

The content of yellow pigments was found to be highest in the central area from 35 to 42°C, and from 500 to 1,000 foot-candles. It gradually decreased toward both low light intensity and temperature, and decreased rapidly toward the killing boundary at high temperature.

The ratio chlorophyll *a* 670/chlorophyll *a*, 682 of a growth pattern similar to that of Figure 1, 48 hours is given in Table I.

Table 1. *The ratio chlorophyll a*, 670/chlorophyll *a*, 682 at different light intensities and temperatures.

Light Intensity in Foot-candles	Temperature in °C			
	15	30	38	44
900	—	2.64	2.86	2.20
400	2.60	3.72	3.40	2.62
200	1.22	2.55	2.36	1.90
75	1.38	2.40	2.24	1.40

The ratio was highest at 400 foot-candles and 30 to 38°C, and from this area it decreased both toward lower and higher light intensity and temperature. It was particularly low when low light intensity was combined with either a high or a low temperature.

Anabaena sp.

Figure 13 shows the growth pattern at the end of an experiment with *Anabaena* sp. (obtained from M.B. Allen). This alga did not develop the striking zones that *Anacystis* did. However, near the low temperature boundary a yellow stripe developed, which by spectrographic analysis was shown to be caused mainly by a destruction of phycocyanin. The killing boundary at high temperature of this species was 44°C at 1,000 foot-candles, and 45°C at 25 foot-candles. At lower temperature the growth boundary was at 28°C at 1,000 foot-candles and at 25°C at 25 foot-candles. The latter boundary, however, did move slowly toward lower temperatures during the experiment, and this species presumably grows fairly well at temperatures below 25°C, particularly at low light intensity.

Figure 14 shows three absorption spectra of *Anabaena* measured from samples all taken at 38°C, but at different light intensities. The curves were made to coincide at the red absorption peak of chlorophyll. At 600 foot-candles the phycocyanin content was somewhat higher than at 950 foot-candles, while the relative amount of yellow pigments had gone down considerably. Finally, at 75 foot-candles, the relative amount of phycocyanin was still higher than in the two preceding samples, and this sample had an extremely low amount of yellow pigments. By the maximum at 575 mμ, the latter curve revealed the presence of phycoerythrin which was not indicated in the two other curves.

The absorption spectra recorded from other samples show that the shape of the curves was very much the same in samples taken from the same light

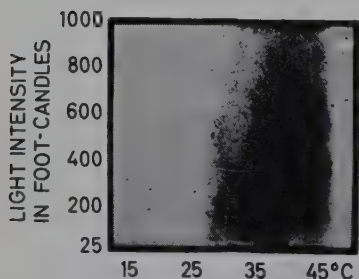
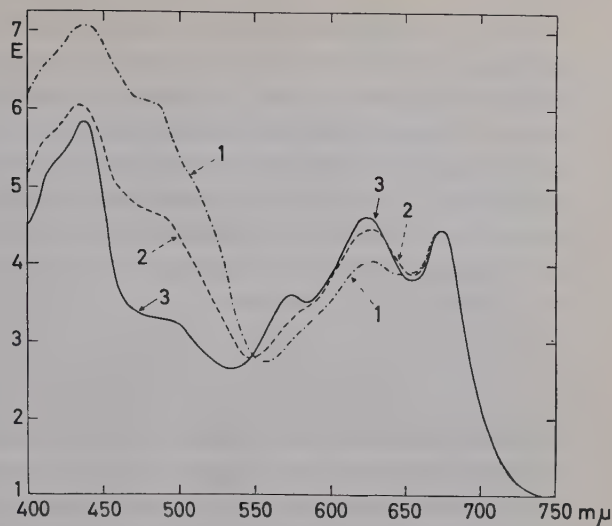


Figure 13. The growth pattern of *Anabaena* sp. after 50 hours growth in crossed gradients of light intensity and temperature.

Figure 14. Absorption curves of three samples of *Anabaena* sp. taken from the same temperature at 38°C, but at different light intensities after 50 hours exposure to crossed gradients of light intensity and temperature of Figure 13. (1) sample from 950 f.c.; (2) sample from 600 f.c.; (3) sample from 75 f.c.



intensity. However, the phycocyanin content was highest at intermediate temperatures around 38°C and lessened somewhat toward both higher and lower temperatures. The maximum around 575 mμ was recorded in all samples taken at low light intensity.

Thus, the relative amount of the different pigments formed in *Anabaena* sp. was particularly dependent upon the light intensity. At high light intensity a very large amount of yellow pigments was formed, but little phycocyanin, and phycoerythrin in such small amounts that it was not revealed in the absorption spectra of living cells. When grown under lower light intensity, *Anabaena* formed much less yellow pigments, but more phycocyanin, and at very low light intensity phycoerythrin was formed in such amount that it was revealed in the absorption spectra of living cells.

Discussion

Liquid Cultures and Agar Growth

It may be suspected that the different characteristic areas which developed under different light and temperature conditions as described in connection with Figure 1, are specific for the growth of *Anacystis* on agar, and that lack of nutrients, for instance, may be one of the reasons for the change that occurred at 30 to 43°C, 400 to 500 foot-candles, where the color changed from fresh green to yellow. However, our experience with liquid cultures strongly suggests that this is a direct temperature-light intensity dependent

effect. If a liquid culture of *Anacystis* is kept at low light intensity, it can be grown over a wide range of temperatures. However, if the light intensity is high, *Anacystis* is very sensitive to minor changes in temperature. In the case of a somewhat low temperature, the culture will turn yellow after a short time; if the temperature is kept within a very narrow range in the higher region, the alga can be maintained in growth with a blue-green color. A slight increase of this temperature will completely bleach the alga evidently due to killing. So far our experiences from liquid cultures are consistent with those from growth on agar.

Chlorophyll a, 670 m μ , and *Chlorophyll a*, 682 m μ

Above we presented evidence for the existence of two spectroscopically different forms of chlorophyll *a* in live *Anacystis*. Curves which have a similar shift in the position of the red absorption maximum, and which also have an indication of a shoulder around 680 m μ have been recorded by French and Halldal (unpublished results) for *Chlorella pyrenoidosa* (Emerson's strain) and for *Chlorella* sp. (TX 71105 of Sorokin and Myers). French (1957) analyzed the first derivative of absorption curves of *Chlorella pyrenoidosa*, grown at different temperatures and light intensities. The curves had features around 670 to 680 m μ which indicated that the absorption in this region of the spectrum is caused by two components. When he integrated the first derivative curve, it coincided well with the absorption curve measured for the same sample in the Beckman spectrophotometer.

The existence of two spectroscopically different types of chlorophyll-*a* *in vivo* has been suggested by Duysens (1952); Yocum and Blinks (1954); and Vorob'eva and Krasnovskii (1956). Vorob'eva and Krasnovskii (*l.c.*) reported a shift in the wavelength of the red absorption peak of leaves grown under different light intensities and temperatures, and suggested that there is an active form with maximum absorption in the red at 670 m μ , which is transformed by strong illumination to an inactive form with a maximum at 678 m μ .

Recently, Shibata (1957), in studies of the protochlorophyll-chlorophyll transformation in intact leaves, showed that protochlorophyll first is transformed, in the light, to a chlorophyll *a* form with maximum absorption in the red at 684 m μ . Then, in the dark, the maximum changes to 673 m μ ; this was in turn succeeded by a final shift to 677 m μ . The latter change may possibly be caused by a relative increase in the 684 m μ form.

French and Young (1956) calculated the curves for the active and inactive pigments of the red algae *Delesseria decipiens* and *Porphyra naiadum* from data of Haxo and Blinks (1950). They found an absorption curve for inactive

pigments of these algae with a reasonable approximation to the *in vivo* absorption spectrum of chlorophyll *a*, and according to their Figures 6—8 (d), with a maximum around 680 m μ , while the absorption of the active pigment has its maximum around 670 m μ in the red part of the spectrum.

Thus, a number of observations suggest the existence of two chlorophyll *a* forms. Only one form of chlorophyll *a* is known in solutions. It is therefore assumed that the two different forms which seem to be present *in vivo* are due to different bindings of chlorophyll to other substances, presumably proteins.

Lately, Latimer (1958) showed that selective scattering will influence many measurements of the absorption spectra of turbid suspensions. By use of a diffusing plate according to the method of Shibata *et al.* (1954) this distortion is greatly diminished. If it is not completely eliminated this may be the reason for the changes that occurred in our measurements. However, as the optical system was the same in all our measurements, and as there seems to be a correlation between change in shape of the curves and conditions on the growth area, the existence of the two chlorophyll *a* forms is probably real.

Complementary Chromatic Adaptation—Intensity Adaptation

In the introduction two of the theories concerning the effect of light on the pigment ratios in algae were summarized: (1) the *complementary chromatic adaptation* theory that the algae assume a color complementary to that of the incident light. This effect has not been subject to analysis in the present investigation; (2) the *light intensity adaptation* theory that the pigments formed are determined by the intensity of the light irrespective of its spectral composition. The present investigation shows that the intensity of "white light" is highly decisive for the pigments formed in both *Anacystis* and in *Anabaena*.

The pigment ratios in *Anacystis* are rather complex and also variable with time (see Figures 11 and 12). It is evident, however, that at low light intensity at all temperatures within the growth area the contents of both chlorophyll and phycocyanin were high, and that it lessened toward higher light intensity except in the narrow stripe around 42 to 45°C where it increased with increasing light intensity. Within the growth area the relative amount of yellow pigments was consistently high where the relative amount of chlorophyll and phycocyanin was low and *vice versa*. This suggests that the photosynthetic activity was low in the center of the growth area where the color changed from fresh green to yellow. The function of the yellow pigments under these conditions might be to act as a light screen, thus

protecting the photosynthetic apparatus from too strong light. The narrow blue-green stripe at high temperature around 42 to 45°C at all light intensities suggests that the photosynthetic apparatus is more effective here than at lower temperatures and high light intensity. The answer to this can only be given by maintaining *Anacystis* under these conditions and then measuring the photosynthetic activity of the alga.

In the experiment with *Anabaena* the pigment pattern was more simple. At low light intensity, the contents of both phycocyanin and phycoerythrin were very high, while the amount of yellow pigments was extremely low. It is well established that the phycobilins are accessory pigments in photosynthesis and it is equally clear that light absorbed by the yellow pigments is less effectively utilized (for review see Blinks, 1954). Lately, it has been shown that β -carotene presumably can actively participate in photosynthesis (Lynch and French, 1957).

The results from the *Anabaena* experiment suggest that two types of adaptation take place: (1) at low intensity accessory pigments such as phycobilins and possibly β -carotene in its active state (suggested by the shoulder at 490 m μ) are formed and give high photosynthetic capacity; (2) with increasing light intensity the relative amount of these pigments was reduced, but more yellow pigments other than that (or those) causing the shoulder at 490 m μ were formed, which may well be yellow pigments inactive in photosynthesis. These yellow pigments possibly act as a light screen in a similar way as β -carotene in oil drops in *Trentepolia* and *Dunaliella* (see Blinks, 1954), thus protecting the photosynthetic apparatus of the alga from high light intensity, as was also suggested for *Anacystis* above.

The experience from laboratory growth of blue-green algae is that they must be kept at a very low light intensity. These algae are in many cases in nature exposed to direct sunlight of the highest possible intensity when floating on tropical water; along the sunny sides of the sea shore; and on desert soil. In the laboratory they will ordinarily die when exposed to only a fraction of natural sunshine. The causes for this may be many. Experiments with *Anacystis* and *Anabaena* showed that both the growth of these algae, and the relative amount of pigments formed, varied from one light intensity — temperature combination to the other. It may be assumed that the conditions in nature favor the formation of some photosynthetically inactive pigments at high light intensity which act as a light screen.

Summary

The blue-green algae *Anacystis nidulans* and *Anabaena* sp. (obtained from M. B. Allen) have been grown on agar in crossed gradients of light intensity

and temperature. A striking, well-defined pattern developed for *Anacystis*, which had great variations from one temperature — light intensity combination to another. *Anabaena* had a more uniform pattern.

Based upon curve analysis of absorption spectra from samples of *Anacystis* taken from different parts of the growth area, the *in vivo* absorption spectra of two chlorophyll *a*, phycocyanin, and yellow pigments have been derived.

A three-dimensional representation of the different pigments formed in *Anacystis* at different light intensities and temperatures at two stages of the development is given.

Phycoerythrin was not revealed in the absorption spectra of *Anacystis*, while the absorption spectra of *Anabaena* from samples taken at low light intensity had a clear maximum at 575 m μ , indicating the presence of this pigment.

I am indebted to Dr. C. S. French, Director of the Department of Plant Biology, Carnegie Institution of Washington, for numerous suggestions during this investigation; and to Dr. Mary Belle Allen for the *Anabaena* culture; and to Mr. R. W. Hart for help in preparing the figures.

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Influence of Preliminary Growth Conditions on Elongation of Roots in Nutrient Solutions. Experiments with *Lupinus albus*

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1. Introduction

During the past 30 years D. I. Macht has published a long series of studies on the effect of human blood serum on the growth of *Lupinus albus* roots.

He has demonstrated in a large number of papers that under certain standard experimental conditions sera from healthy persons inhibit the growth of lupine roots. With some human diseases the inhibitions is, according to his investigations, more pronounced than when sera from healthy persons are used.

Macht's first paper on this subject came out in 1922. In 1941 he published a comprehensive statistical assessment of his method. Later on, he has prepared several further relevant papers. The latest one, which appeared in 1955, deals with the toxicity to lupine roots of urine from cancer patients.

Macht's standard experimental conditions comprise *imbibition* overnight in tap water with subsequent germination and preliminary growth in washed Sphagnum having a water content of 70—80 %, "by weight," for 3 or 4 days at room temperature. When the roots have reached a length of 35—50 mm. they are considered fit for experiments. The experiments are performed by submerging the roots in a nutrient solution (according to Shive 1915) partly without and partly with an addition of 1 % newly prepared serum. The elongation in 24 hours at 15°C is measured, and on the basis of the average value from control experiments and serum experiments (generally, 10 roots are used in each test) he calculates the

index *ad modum* Macht, or index of elongation, by expressing the elongation in the solutions with sera as a percentage of the elongation in the control experiment:

$$I_E = \frac{100 \times E_{\text{serum}}}{E_{\text{Shive}}}$$

According to Bertossi (1953) the lupine test was originally developed by Benedicenti and De Toni in 1901. Bertossi has investigated the sensitivity of the method to diphenyl acetic acid and finds in a statistically well-founded work that the use of twenty about 37 mm. long roots in each experiment gives sufficiently reliable results. In his opinion, the reason why the lupine test has acquired the reputation of being a poorly sensitive method is solely that it is often applied without being adequately founded statistically.

In later years the present author has occupied himself with investigations on the content of phytohormones in human blood with the use of Macht's method. The material here submitted comprises an analysis of the technique from a plant-physiological point of view. Efforts have been made to throw light on some facts which have proved important for the achievement of reproducible results in tests with sera and in control experiments, when investigations extend over a long time.

In the statistical treatment of the index values, 95 % confidence intervals are used throughout the paper. These intervals are calculated according to Yule and Kendall, p. 299, by the formula (modified):

$$\bar{S} \pm t_{.05, I_E} = 100 \cdot \frac{E_{\text{serum}}}{E_{\text{Shive}}} \sqrt{\left(\frac{t_{.05} \bar{S}_{\text{serum}}}{E_{\text{serum}}} \right)^2 + \left(\frac{t_{.05} \bar{S}_{\text{Shive}}}{E_{\text{Shive}}} \right)^2}$$

2. Technique

Material. In accordance with Macht, seeds of the size 9—11 mm. of a bitter *Lupinus albus* variety were used. The seeds are imported from Italy and are never more than 2 years old when used.

Imbibition. The dry seeds are imbibed for 24 hours in distilled water at 20°C. Under these conditions the imbibition is at its maximum (Bertossi 1953).

Germination and preliminary growth. Cylindrical glass jars with lids, as shown in Figure 1, are used for the cultivation. The jars are 14.5 cm. deep and 11.5 cm. in diameter, and are filled with culture medium up to 1—2 cm. from the brim. In such a jar a good 80 seeds can be put for germination simultaneously, the seeds being placed beside each other on top of the medium and with the hilum pointing downward. After sowing, the seeds are overspread with a loose layer of the medium about 1 cm. thick, and, finally, each jar is covered with a lid, not completely preventing access of air. Thereby evaporation is greatly impeded.

Imbibition and germination as well as growth tests and root measurements take place in one and the same basement room, where the temperature is kept at 20°C ± 0.5 by a contact thermometer and a relay, connected with a small electric heater.

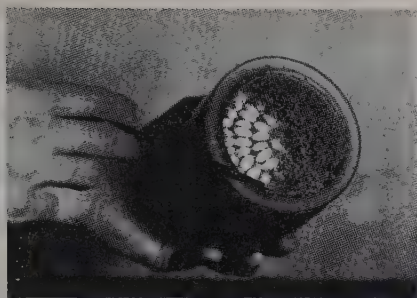


Fig. 1.



Fig. 2.

Figure 1. *Germination jar with seedlings of *Lupinus albus* in sphagnum.*

Figure 2. *Set-up for cultivation in nutrient solution.*

The constancy of this temperature is very important for the reproducibility of the experiments. The temperature is checked constantly by a thermograph. The relative humidity in the room is around $70\% \pm 15$. Germination and all other growth takes place in total darkness. The period of preliminary growth is generally 60 hours.

Culture in nutrient solution. After germination and growth in a preliminary medium the roots are used for phytohormonal tests and placed in nutrient solutions for further growth. For this purpose racks with twenty 10 cm. deep cylindrical test tubes of 12 mm. diameter are used (Figure 2). The tubes are calibrated, so that the young plants can be put into them directly, the cotyledons resting on the brims of the tubes. The tubes are filled with nutrient solution right up to the brim.

In accordance with Shive 1915, the nutrient solution is made up of 10.4 ml. 0.5 M $\text{Ca}(\text{NO}_3)_2$, 30 ml. 0.5 M MgSO_4 , and 36 ml. 0.5 M KH_2PO_4 , diluted to 2000 ml.; addition of ferric phosphate, however, as normally applied to Shive's solution, is not used. In this solution the roots are allowed to grow for exactly 24 hours at $15^\circ\text{C} \pm 0.1$, the tubes being placed in an air-cooled incubator or in a water bath as

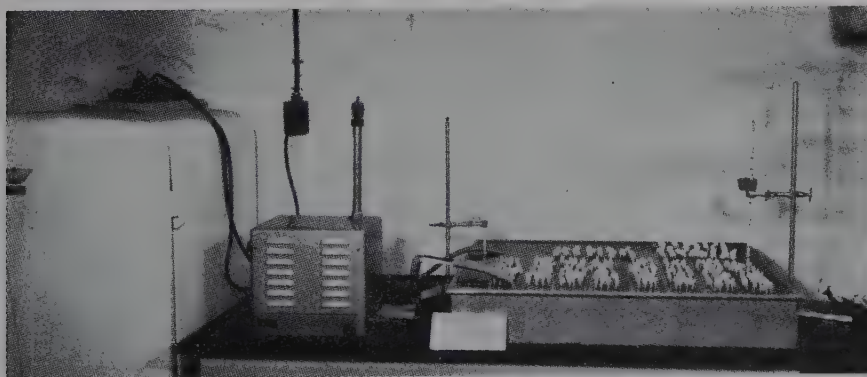


Figure 3. *Set-up of nutrient solution test tubes in water bath. The water is circulating through the refrigerator and kept at $15^\circ\text{C} \pm 0.1$.*



Figure 4. *Measuring of roots under a hand lens with a slide caliper. Accuracy is held to plus or minus 1/10 mm.*

shown in Figure 3. The fact that in the latter case the cotyledon temperature becomes slightly higher than 15° is of no consequence to the growth of the roots (Rønnike 1957b).

Measuring of root lengths. The actual root measurements are made directly under a faintly illuminating table lamp (about 150 lux). The more expedient method is to use a big hand lens and a slide caliper as shown in Figure 4. In nutrient solution experiments, before measuring the initial length, the roots are cleaned of the preliminary growth medium, being first brushed with a soft paint brush (Figure 5) and, next, rinsed several times in distilled water. The length of the root is measured from the root tip to the junction with the hypocotyl. Some practice is required to distinguish the junction of stem and root. This junction is, however, always plainly and sharply defined, if only care is taken to ensure a definite angle of incidence of the light. Furthermore, it is important always to measure the root from the same point of the boundary line, viewed in relation to the point of attachment of the cotyledons, the boundary across the root sometimes having an oblique direction.

3. Media for Germination and Preliminary Growth

It may be assumed to be very important to have a medium for germination and preliminary growth which is unchanged from experiment to experiment and can be defined exactly. Some of the factors which are essential for the growth of roots in artificial or natural media are still unknown. To make an attempt at keeping these factors constant, so that the conditions of culture may be standardised and reproducible, care will have to be taken, in the first place, to ensure that the stock material for preparation of the cultivation medium is as homogeneous as ever possible. Next, the water content of the medium must be exactly the same from experiment to experiment. The water content should as far as possible be kept constant throughout the preliminary growth period and not only at the time when the seeds are sown.

The water content of the preliminary culture medium (in the following always expressed as a percentage of absolutely dry matter) is very important to the growth of the roots. It has been demonstrated previously that a boundary value of the water content exists, below which water-imbibed



Figure 5. *Cleaning the root of sphagnum* (Photos Figures 1, 2, 4, and 5: L. Fosgård).

lupine seeds display growth of the roots exclusively, and above which both root- and stem-tissue will grow (Rønnike 1957a). The water content of the medium in which preliminary growth takes place is strangely enough also very important to the subsequent growth in the liquid medium. This after-effect is expanded upon in a later section of this paper.

Much work has been made to find the most suitable method of procuring test material of uniform quality. For instance, cultivation in humid air in vertical ducts in plaster blocks or between filter paper, and cultivation in various types of saw dust and raised-bog sphagnum, have been tried. A few details of this work will be made the subject of further mention.

Specially designed germination stands in which each individual seed was surrounded by moist filter paper, seemed to be the most appropriate for preliminary cultivation in humid air. The object of this technique was to obtain growth in a medium where the roots would be kept perfectly clean so as to be directly useable for liquid-medium experiments. The method was as simple as could be, as far as reproducible conditions of growth were concerned, but, after all, it was found not to be as suitable as the sphagnum method, which is accounted for in the following. Despite all endeavours to keep filter paper and germination equipment microbiologically clean, 10 to 20 % of the seedlings were found always to be infected, mostly by fungal attack. On one occasion all roots were affected by an acute, lethal bacteriosis attacking the roots right beneath the hypocotyl. Such infections never occurred with the cultivation in sphagnum. Incidentally, the filter-paper method was rather cumbersome, costly, and time-devouring, and was therefore abandoned.

After trying out of the abovenamed media and methods of germination and preliminary growth, the sphagnum preparation »Nodampoff», supplied by The Meyer Seed Company, Baltimore 2, Md., USA, was ultimately adopted. This is a preparation essentially of the same type as the one used by Macht.

The Nodampoff sphagnum preparation has been used partly directly and partly, and by far predominantly, in washed condition. The washing is best carried out in a stainless steel container to which running water of about 70°C is admitted at the bottom and passed on to be discharged at the top, where sphagnum is retained by a piece of wire gauze. After the washing, which takes a couple of hours, the

material is first squeezed or sucked dry, and next redried in a cabinet through which air of constant temperature of 40° and of constant humidity (40 %) is blown for some 30 hours. Thereafter the water content is about 10 %. After drying, the sphagnum material is sifted through a screen so that the particle size is estimated to be approximately the same as in unwashed sphagnum. The specific gravity of washed sphagnum is, however, as a rule greater than that of unwashed sphagnum, and the washed material is darker than the unwashed.

Before being used as growth medium, the sphagnum is adjusted to the requisite water content by admixture of water in big enamelled buckets. After intimate stirring, the buckets are sealed airtight and left for about 24 hours to ensure a perfectly uniform water concentration throughout the material. Immediately before application samples are sometimes drawn for exact moisture determination.

As will be demonstrated in the following section, it is very important to the rate of growth of the root whether washed or unwashed sphagnum is used. The effect also manifests itself during subsequent growth in liquid medium. Little is known about the actual happenings during the washing, beyond the fact that the concentration of certain substances in the medium is, of course, reduced. Perhaps a change is also produced in the microbiological flora and fauna, since the washing is done with comparatively warm water. The diffusion pressure deficit in washed and unwashed sphagnum of the same water percentage may also be assumed to be different owing to different colloidal conditions of the two media, and a change in the average particle size caused by the washing may be of some consequence. A detailed investigation of these problems is, however, deemed to be beyond the scope of this paper.

In the next two sections (4 and 5) an account is given of the elongation of lupine roots during preliminary growth in sphagnum under various conditions, and during subsequent growth in nutrient solutions. Finally, section 6 accounts for the obtained relative constancy of the rate of growth of lupine roots in nutrient solution over a period of one whole year, when the roots are cultivated under conditions standardized in respect of preliminary growth as well as of growth in liquid medium.

4. Growth of Lupine Roots in Sphagnum

Figure 6 shows curves representing the elongation of lupine roots in washed Nodampoff sphagnum with different water content. The uppermost curve corresponds to 300 % water, the intermediate one to 75, and the lowermost one to 60 % water; all at 20°C. For each individual value measured, 95 % confidence intervals are given on the curve, the size of these

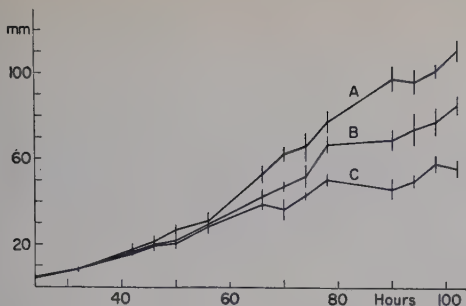


Fig. 6.

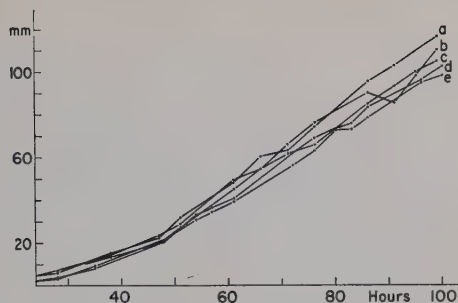


Fig. 7.

Figure 6. Growth of roots of *Lupinus albus* in washed sphagnum with A: 300, B: 75, and C: 60 % water.

Figure 7. Growth of roots of *Lupinus albus* in washed sphagnum with increasing water percentages; a: 300, b: 400, c: 600, d: 800, and e: 1000 % water.

intervals corresponding to the lengths of the lines perpendicular to the curves. It will be seen how the values overlap to the left on the curve, whereas after a good 60 hours' growth significant differences occur between the lengths of the roots in the different media.

The theoretical polynomial of the 2nd degree for the indicated curve of growth in washed sphagnum with 300 % water is $\hat{Y} = -1.39 + 1.131 X + 0.0040 X^2$, where a curved regression is thus presumed to exist. From the equation there appears to be positive acceleration in the rate of growth. The probability of this event can, according to Snedecor (1952, p. 454), be directly calculated to lie between 90 % and 95 %.

Similarly, the equation of the 2nd degree for the curve of growth in washed sphagnum with 75 % water is $\hat{Y} = 0.81 + 0.907 X + 0.0023 X^2$, and for 60 % water $\hat{Y} = 1.81 + 0.954 X - 0.0033 X^2$. In none of these cases can any significant flexion of the curves be demonstrated, and the rate of growth may therefore be said to be constant.

Figure 7 presents curves of growth in washed sphagnum with water percentages above 300 %. As will be seen, there is an optimum of the rate of growth in sphagnum with 300 % water, the rate of growth being lower in sphagnum with lower percentages of water (Figure 6), but no higher in sphagnum with water percentages from 300 up to 1000.

In Figure 8 four curves of growth calculated on the basis of experiments are represented, two for washed Nodampoff (solid lines) and two for unwashed Nodampoff (dotted lines). The two upper curves show the growth in sphagnum with 300 %, the two lower ones the growth in sphagnum with 75 % water. The equations for the solid-line curves are as indicated above;

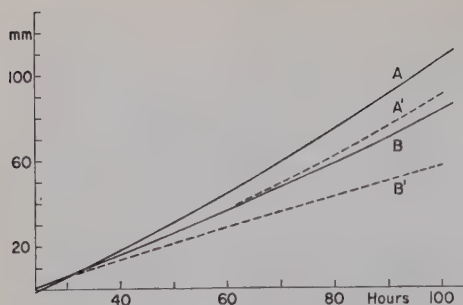


Figure 8. Calculated curves of growth (polynomials of the 2nd degree) for roots of *Lupinus albus* in washed sphagnum (solid-line curves) and unwashed sphagnum (dotted curves). A and A': 300 % water, B, and B': 75 % water.

for the dotted-line curves they are, respectively, $\hat{Y} = 0.96 + 0.841 X + 0.0044 X^2$ for unwashed sphagnum with 300 % water, and $\hat{Y} = 3.72 + 0.759 X - 0.0004 X^2$ for unwashed sphagnum with 75 %.

The Figure shows that the same water percentage gives widely different rates of growth in the two types of medium. For instance, the growth in washed sphagnum with only 75 % water is approximately comparable with the growth in unwashed sphagnum with 300 % water. The disparity in rates of growth found for the roots in washed and unwashed sphagnum, respectively, also manifests itself, and very distinctly at that, as an after-effect, if the roots are transferred to liquid medium.

For the standardized technique described in section 6, roots cultivated preliminarily in washed sphagnum with 300 % water are always used. This water percentage has been selected because, as already mentioned, the rate of growth therein was found to be at the optimum value.

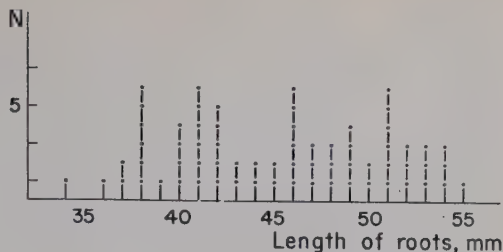
It may be added that it is impossible to distinguish macroscopically between roots from, say, sphagnum with 100, and sphagnum with 600 % water.

5. Growth of Lupine Roots in Liquid Medium

Figure 9 illustrates in a dot diagram the distribution of the lengths of lupine roots after some 60 hours' growth in washed Nodampoff with 300 % water. The roots in the diagram all originate from the same germinating jar, picked at random, with 97 % germination. The average length is 44.8 mm. and variation is from 35 to 55 mm. The diagram is representative of the uniformity at which the test material can be provided.

The increment of the roots in liquid medium might now be imagined as dependent on the root length at the time of transfer to the nutrient solution (the *initial length*). For investigation of this question, all roots from experiments in the months of January, February, and March 1956 were collected in groups with the same initial lengths, and the average increment (in 24

Figure 9. Dot diagram showing the distribution of initial lengths of roots in one germination jar like that of Figure 1.



hours at 15°) was calculated for each individual group. All these roots had been subject to uniform preliminary treatment. The result is plotted on the curve in Figure 10. The coefficient of regression for this curve is $+0.17$, and the probability that this differs from 0 lies between 99 and 99.5 % as an expression for the fact that the growth in Shive's solution increases with the initial length for roots with the same preliminary growth period.

However, this increase is but slight. In the preparation of each individual test, care should, anyhow, be taken to ensure that the roots are distributed as uniformly as possible, in respect of initial lengths, among the control experiment and the various solution with growth-inhibitory properties. In the present work only initial lengths between 40 and 50 mm are used in order to reduce to a minimum the effect of these quantities on the test results.

When this condition with regard to the initial lengths of the roots has been established it has further to be investigated whether the elongation of the lupine roots in Shive's solution takes place in such a way that the statistical standard distribution laws may be applied. If these are valid, it must then be investigated whether the standard deviation and mean error are of such an order that the experimental technique is practicable.

In Figure 11 six arbitrarily selected probit curves for elongation of lupine roots in Shive's solution (E_{Shive}) are plotted. It will be noted how the observations in each experiment distribute themselves along straight lines with almost equal slopes as an expression for the fact that the distributions are normal, and that, with very great approximation, they have the same

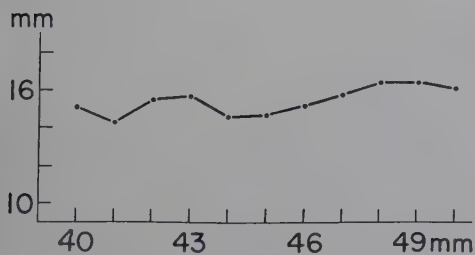


Figure 10. The effect of the initial length on the average elongation in Shive's solution of roots preliminarily grown under standard conditions (see text of Figure 12).

Probits

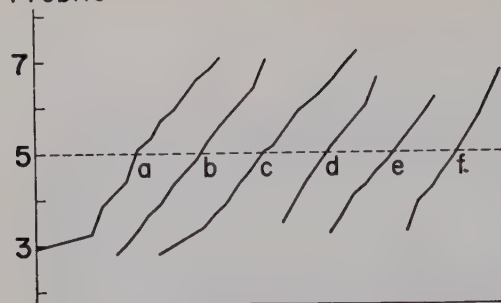


Figure 11. Probit analyses of elongations of roots of *Lupinus albus* in Shive's solution, six experiments, a—f, picked at random.

standard deviation. The arithmetical operations that are applicable for the normal distribution may therefore also be applied to the growth measurements of lupine roots in liquid medium.

To find out what period of preliminary growth may be considered the best, an investigation was made of the variations of the E_{Shive} values for roots of different ages and consequential different initial length average (Table 1).

Table 1. Elongation in Shive's solution (E_{Shive}) of *Lupinus albus*-roots, preliminarily grown in washed sphagnum (*Nodampoff*) with 300 % water. Experiments with varied duration of preliminary growth.

Experiments	Number of roots (n)	Duration of preliminary growth (hours)	Average initial length (mm)	$E_{\text{Shive}} \pm t_{.05} s_{\bar{x}}$ (mm)	t	P	Significance of differences
26—27/8 1957 (a)	50	60	45.3	18.8 ± 1.3	2.276	$0.025 < P < 0.050$	+
	50	50	32.2	16.9 ± 1.4			
10—11/10 1957	40	60	45.4	18.7 ± 1.7	0.697	$0.400 < P < 0.500$	—
	40	50	31.2	19.5 ± 1.6			
18—19/10 1957 (a)	40	60	45.3	15.9 ± 2.1	1.216	$0.200 < P < 0.400$	—
	40	50	28.9	17.5 ± 1.6			
21—22/11 1957	40	60	43.7	18.7 ± 1.3	1.268	$0.200 < P < 0.400$	—
	40	50	29.9	19.8 ± 1.2			
29—30/12 1957	40	60	43.2	22.0 ± 1.5	4.608	$P < 0.001$	+
	37	50	28.1	19.1 ± 1.1			
6—7/1 1958	40	60	44.7	22.0 ± 0.9	5.139	$P < 0.001$	+
	40	50	28.8	19.0 ± 0.8			
13—14/1 1958	39	60	46.5	18.4 ± 0.8	2.687	$0.005 < P < 0.010$	+
	40	50	36.3	17.1 ± 0.6			
26—27/8 1957 (b)	35	71	70.0	12.2 ± 1.0			
18—19/10 1957 (b)	40	72	69.2	11.2 ± 1.6			

Table 2. *Elongation in Shive's solution (E_{Shive}) of roots preliminarily grown in sphagnum with varied water content.* 1) Comparison between roots with identical duration of preliminary growth (and, consequently, different initial lengths of roots). 2) Comparison between roots with different duration of preliminary growth (but identical length of roots). 3) Comparison between roots preliminarily grown in washed or unwashed sphagnum.

Experiments	Sphagnum washed	Water-percentage in sphagnum	Number of roots (n)	Duration of preliminary growth (hours)	Average initial length (mm)	$E_{\text{Shive}} \pm t_{.05} S_{\bar{x}}$ (mm)
14—15/2 1956	+	50	28	Ca. 70	29.8	8.4 ± 1.0
	+	75	30	" 70	35.1	9.9 ± 0.8
	+	100	31	" 70	40.4	10.9 ± 1.0
	+	125	31	" 70	43.8	12.4 ± 1.0
	+	150	32	" 70	40.8	11.3 ± 1.1
	+	300	30	" 70	46.8	15.0 ± 1.4
21—22/2 1956	+	100	26	" 70	39.8	11.6 ± 1.2
	+	300	32	" 70	45.4	15.3 ± 1.1
12—13/8 1957	+	75	50	74	50.1	7.7 ± 1.5
	+	300	50	57	44.5	16.2 ± 1.6
	—	75	50	76	44.4	2.6 ± 0.9
	—	300	50	58	45.0	9.1 ± 1.8
19—20/8 1957	+	75	50	66	44.9	8.7 ± 1.7
	+	300	50	62	45.5	16.2 ± 1.2
	—	75	50	72	44.4	3.1 ± 0.9
	—	300	50	60	44.7	8.7 ± 1.5

In the experiments carried out on August 26th—27th (a), December 29th—30th, 1957, January 6th—7th, and 13th—14th, 1958, the growth of the initially shorter roots was slightly smaller than that of the initially longer ones, whereas in the experiments on October 10th—11th, October 18th—19th, and November 21st—22nd no difference was found. It is justifiable to assume that a shorter period of preliminary growth gives the same E_{Shive} value, since the differences found in the experiments on August 26th—27th (a), December 29th—30th, and January 1958 may be presumed to be due to date variations (*inter* experiment variations) in the E_{Shive} values. The differences found in these experiments are smaller than the range applying to the E_{Shive} values from one experiment to the next as appears from Figure 12.

A composite analysis of variance (calculated according to Snedecor, p. 296) also shows that there is no significant difference between the E_{Shive} values for roots with different periods of preliminary growth, when several experiments are involved. (From Table 1: $F=1.185$; $P < 70\%$.)

On the other hand, a period of growth longer than 60 hours, and consequently a longer initial length, gives plainly far lower E_{Shive} values; cf. the experiments of August 26th—27th (b), and October 18th—19th (b), 1957.

Table 3. *Elongation in Shive's solution (E_{Shive}) of roots preliminarily grown in washed sphagnum with water contents equal to and above 300 %.*

Experiments	Water-percentage in sphagnum	Number of roots (n)	Duration of preliminary growth (hours)	Average initial length (mm)	$E_{\text{Shive}} \pm t_{.05} s_{\bar{x}}$ (mm)	t	P	Significance of difference
22—23/9 1957	400	40	60	43.7	20.0 ± 0.8			—
	600	40	60	43.9	20.3 ± 0.7			
30/9—1/10 1957	300	40	61	46.1	16.7 ± 1.6	2.499	$0.010 < P < 0.025$	+
	400	40	61	45.9	18.0 ± 1.0			
	600	40	61	44.8	19.1 ± 0.8			
7—8/10 1957	300	40	60	45.2	15.6 ± 1.5	4.802	$P < 0.001$	+
	600	32	60	45.1	18.7 ± 0.9			
	800	32	60	45.3	20.2 ± 1.0			
15—16/10 1957	300	40	60	45.6	18.3 ± 1.2	2.020	$0.025 < P < 0.050$	+
	1,000	40	60	41.8	19.9 ± 1.1			
22—23/10 1957	300	40	60	44.2	17.2 ± 1.1	3.003	$0.001 < P < 0.005$	+
	1,500	33	60	49.4	19.4 ± 0.9			
12—13/12 1957	300	40	60	43.9	21.8 ± 0.9	4.298	$P < 0.001$	+
	800	36	60	44.8	19.1 ± 0.9			
19—20/12 1957	300	36	60	43.1	20.8 ± 1.0			—
	800	36	60	43.7	21.2 ± 0.9			
30—31/12 1957	300	40	60	43.2	22.0 ± 1.5	1.613	$0.100 < P < 0.200$	—
	800	39	60	39.8	20.6 ± 1.6			
13—14/1 1958	300	39	60	46.5	18.4 ± 0.8			—
	800	34	60	45.7	18.5 ± 0.7			

The water content in the culture medium during the preliminary growth is a factor of very great importance to the elongation of roots in the liquid medium, as may be seen from Table 2. This Table also gives E_{Shive} values of roots preliminarily grown in washed and unwashed sphagnum of different water contents. It will be observed how E_{Shive} rises on an increase of the water content in the preliminary growth medium, and this rise is evident both when comparing roots with identical duration of preliminary growth, and consequently different initial lengths, and when comparing roots with different duration of preliminary growth but identical initial length.

When Table 2 is read together with a consultation of the curves of growth in sphagnum (Figure 8), good agreement is found between the rate of preliminary growth in sphagnum and the simultaneous E_{Shive} values for roots from media of different moisture contents.

Table 3 gives E_{Shive} values for roots grown preliminarily in sphagnum with water contents of 300 % and up. A significant difference between the E_{Shive}

Table 4. *Elongation in Shive's solution (E_{Shive}) of roots of identical initial length, preliminarily grown at different temperatures in washed sphagnum with 300 % water.*

Experiments	Number of roots (n)	Temperature at preliminary growth ($^{\circ}\text{C}$)	Duration of preliminary growth (days)	Average initial length (mm)	$E_{\text{Shive}} \pm t_{.05} s_{\bar{x}}$ (mm)
6—7/5 1957	32	10	11	44.4	14.4 ± 1.3
19—20/5 1957	32	5	25	44.3	9.7 ± 1.9

values for roots from preliminary media with 300 % water and those for roots from media with more water is often noticeable within a single experiment. A composite analysis of variance, however, shows that there is no significant difference when several experiments are included. ($P < 90\%$)

The growth in Shive's solution is dependent on the nature of the preliminary growth medium also when other factors than water content are considered. This appears from a comparison of the values for the experiments of August 12th—13th and August 19th—20th (Table 2), which shows how E_{Shive} values of roots grown in washed and unwashed sphagnum of the same water content are of widely different magnitudes. Compared in another way, the elongation of roots from unwashed sphagnum with 300 % water corresponds to the elongation of roots from washed sphagnum with only 75 % water, which is in perfect accordance with the results shown in the curves of Figure 8.

Table 4 shows E_{Shive} values of roots grown preliminarily at temperatures lower than the usual 20°C . It will be seen that roots cultivated in sphagnum at 10° and 5° have elongations in Shive's solution of 14.4 mm. and 9.7 mm., respectively. The ordinary standard value (*i.e.* E_{Shive} of roots germinated in whashed sphagnum with 300 % water at 20°) was in the period when the experiments were carried out found to be around 18—21 mm. Thus, the temperature in the period of preliminary growth also has some effect on the subsequent growth in liquid medium.

6. Obtainable Constancy

The curve in Figure 12 shows fluctuations in the E_{Shive} values throughout the year 1956 and in the early months of 1957. All roots used for measurements shown in this curve were cultivated under uniform, standardized

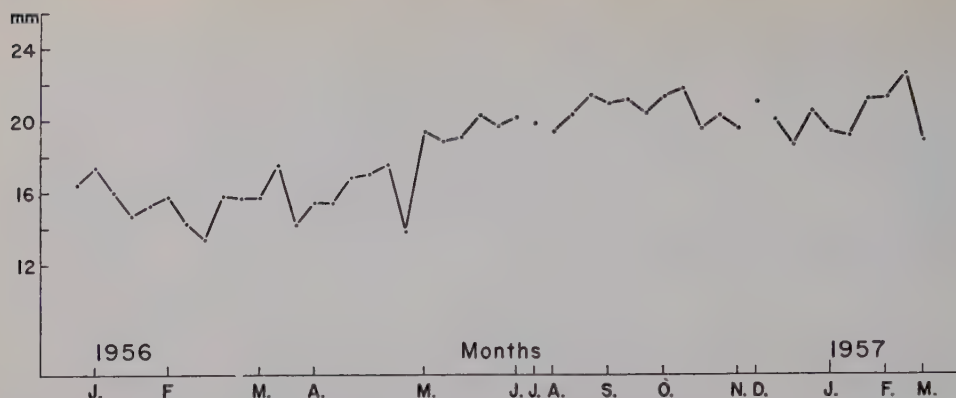


Figure 12. Variation of the standard value over a period of one whole year. The standard value (E_{Shive}) means the average elongation in Shive's solution in 24 hours at 15°C of roots of *Lupinus albus* L. cultivated preliminarily under standard conditions (about 60 hours' growth at 20°C in washed sphagnum with 300 % water).

conditions (washed Nodampoff sphagnum with 300 % water, preliminary growth for about 60 hours at 20°C , initial lengths between 40 and 50 mm).

The coefficient of regression for the first six months of 1956 is 0.173, and the probability that the increase of the values of this period is real is greater than 99.95 %. The mean of the Shive elongations is 16.6 ± 0.8 mm. During the latter half of 1956 and in January–February 1957 a coefficient of regression of 0.02 is had, which does not differ significantly from 0 as an expression for the fact that the values fluctuate erratically about the average value 20.1 ± 0.6 mm. This latter relative constancy should be compared with that of the wheat root test of Hansen (1954) and Burström (1953).

Nothing can be said for certain about the reason for the increase in the values during the first six months of 1956. The previous use of the cultivation medium as germination medium may have been important. Before each new experiment the medium is, of course, dried and adjusted to standard percentage of water. Previous application of the sphagnum once or twice as preliminary growth medium produces an increased elongation in the Shive solution, whereas more repeated use results in decreasing E_{Shive} values. Systematic investigations of this phenomenon, however, have not been carried out.

In addition to the conditions present in the preliminary growth medium, the conditions of the seeds, *e.g.*, the age of the seeds, may, of course, be of importance for the growth rate. Moewus points out (1949, p. 123) that the rate of growth of cress roots declines after the third year counted from the harvest of the seeds, and that the germination percentage in darkness is not complete until the seeds have reached the age of six months.

Table 5. *Elongation in Shive's solution (E_{Shive}), compared with elongation in distilled water ($E_{\text{dist.}}$) of roots preliminarily grown in washed sphagnum with 300 % water.*

Experiments	Number of roots (n)	Average initial length (mm)	$E_{\text{Shive}} \pm t_{0.05} s_{\bar{x}}$ (mm)	$E_{\text{dist.}} \pm t_{0.05} s_{\bar{x}}$ (mm)	Relative growth I_E
12—13/11 1956	40	48.3	19.4 ± 0.9	22.7 ± 1.2	100 ± 5
	40	48.7			117 ± 8
19—20/11 1956	40	48.5	20.1 ± 1.2	23.0 ± 1.2	100 ± 6
	40	47.7			114 ± 9
26—27/11 1956	40	45.5	19.4 ± 0.9	22.1 ± 1.1	100 ± 5
	40	45.5			114 ± 8
22—23/12 1956	40	45.3	20.9 ± 1.0	23.7 ± 1.4	100 ± 5
	36	45.7			113 ± 9
14—15/1 1957	40	47.8	18.5 ± 1.0	20.0 ± 1.1	100 ± 5
	40	47.9			108 ± 8
4—5/2 1957	40	46.1	19.0 ± 1.0	21.5 ± 1.1	100 ± 5
	40	45.5			113 ± 8

Åberg says in his treatise on flax roots response on growth substances (1954) that "it is impossible to obtain an absolute constancy in the growth of the control roots lasting over a period of several years, during which different seed batches have to be used. The seeds of a single batch do also undergo endogenous changes, which may cause considerable differences in germination percentage and in the early growth of the seedlings". These observations agree very well with the evidence brought forward in the present paper.

7. Sensitivity of Lupine Roots in Test work

Some examples of the sensitivity of lupine roots are given below. Table 5 shows the growth of roots in distilled water, as compared with the growth in Shive's solution. It will be seen that in all the tests the elongation in distilled water is larger than in the nutrient solution. The test of January 14th—15th, 1957, is an exception. A composite analysis of variance taking account of the variation from experiment to experiment shows that the difference between the growth in Shive's solution and the growth in distilled water can be reproduced from experiment to experiment with a certainty of above 95 but below 99 %.

This is in perfect agreement with Ekdahl's investigations of the rate of growth of wheat roots in bidistilled water compared with the rate of growth

Table 6. *Elongation of roots in Shive's solution with and without L- and D-chloramphenicol (1/1000). Experiments with roots preliminarily grown in washed sphagnum with 300 ‰ water.*

Experiments	Medium	Number of roots (n)	Average initial length (mm)	Elongation $\pm t_{.05} s_{\bar{x}}$ (mm)	Relative growth (I_E) $\pm t_{.05} s_{\bar{x}}$
9-10/9 1957	Shive's solution	68	45.0	16.3 ± 1.1	100 ± 7
9-10/9 1957	+ L-chloramphenicol	28	45.0	7.9 ± 0.8	49 ± 6
9-10/9 1957	+ D-chloramphenicol	28	45.3	11.0 ± 1.3	68 ± 9

in nutrient solution (1957). In the former case he finds a rate of growth of 21.8 ± 0.36 mm. (67 ‰ confidence intervals), in the later 20.3 ± 0.25 mm. The index is 107.5 ± 1.41 ‰ (24 hours at 20°C). "This relation varied in the experimental series. In one series it was 103 per cent only, and in another 111 per cent. The high growth in bidistilled water was observed on the first day only. On the following two days the growth in this medium was rapidly lowered".

Table 6 shows growth in D-chloramphenicol, compared with growth in L-chloramphenicol, both in the concentration 1000 $\mu\text{g}/\text{ml}$ of Shive's solution (1/1000). The chloramphenicol preparations were placed at disposal by Lepetit, of Milan. The growth is depressed in both cases, but mostly in the solution with L-chloramphenicol. Assuming that the preparations are pure, there should thus be a distinct difference between the root-growth inhibitory properties of these two preparations. In the later growth of the roots, how-

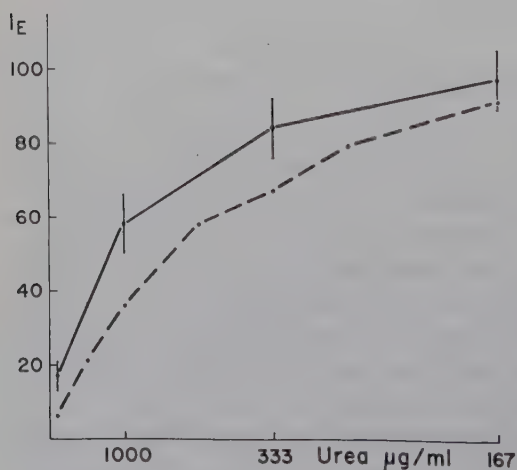


Figure 13. *Elongation of roots of Lupinus albus in various concentrations of urea, expressed as percentages of the standard value (E_{Shive}) at corresponding test dates. Dotted curve: Macht's data of 1955.*

Table 7. *Elongation of roots in Shive's solution without (E_{Shive}) and with urea (E_{urea}). Concentration of urea 500 $\mu\text{g}/\text{ml}$. Experiments with roots preliminarily grown in washed sphagnum with different water contents.*

Experiments	Water percentage in sphagnum	Number of roots (n)	Duration of preliminary growth (hours)	Average initial length (mm)	$E_{\text{Shive}} \pm t_{.05} s_{\bar{x}}$ (mm)	$E_{\text{urea}} \pm t_{.05} s_{\bar{x}}$ (mm)	Relative growth (I_E) $\pm t_{.05} s_{\bar{x}}$
9—10/9 1957 {	300	68	60	45.0	16.3 ± 1.1	100 ± 7	81 ± 8
	300	40	60	45.1			
2—3/9 1957 {	300	40	66	51.3	14.7 ± 1.6	100 ± 11	62 ± 12
	300	40	66	51.9			
7—8/10 1957 {	300	40	60	45.2	15.6 ± 1.5	100 ± 10	65 ± 12
	300	40	60	46.3			
9—10/9 1957 {	50	40	70	44.8	5.7 ± 1.9	100 ± 33	68 ± 40
	50	40	70	45.4			
2—3/9 1957 {	100	52	71	51.1	8.2 ± 1.7	100 ± 21	63 ± 22
	100	52	71	51.2			
2—3/9 1957 {	500	50	65	49.9	17.3 ± 0.8	100 ± 5	70 ± 6
	500	50	65	51.1			

ever, it has been impossible to observe any difference between the lateral root formation effect of these two isomeric compounds, such as found by Semenza.

The curve in Figure 13, finally, shows the growth-inhibitory properties of urea in various concentrations (1/100, 1/1000, 1/3000, and 1/6000, corresponding to 10,000, 1000, 333, and 167 $\mu\text{g}/\text{ml}$. of Shive's solution). As previously, the vertical lines indicate 95 % confidence intervals for the individual experimental results. The dotted line indicates Macht's data of 1955.

Table 7 shows the inhibition of 500 μg . urea/ml. of Shive's solution, but, in addition to the inhibition to roots from sphagnum with 300 % water as in Figure 13, also inhibition to roots cultivated preliminarily in sphagnum of other water percentages is indicated.

All responses of the roots are expressed as percentages of the growth in the corresponding pure Shive growths (I_E). The urea concentration used in the tests in Table 7 has been chosen because its inhibition is of approximately the same order as that of standard serum, but, while standard serum is a liquid of variable composition, the urea solution can be defined exactly.

It will be noted how the values for roots from sphagnum with 300 % water fluctuate about 70 % (81, 62, and 65 % of the growth in Shive's solution). It can also be seen how roots grown preliminarily in media with contents of 50, 100, and 500 % water show no inhibition differing from that of roots from sphagnum with 300 % water (68, 63, and 70 % of comparable E_{Shive} values).

Table 8. *Elongation of roots in Shive's solution without (E_{Shive}) and with serum (E_{serum}). Serum I, used for experiments 1—8, is prepared from one and the same person at two different dates. The inhibitory effect of this serum corresponds to the average level most often found. Serum II originates from another person and shows a higher inhibitory effect than serum I.*

Experiments 1956.	Water per- centage in sphagnum	Number of roots (n)	Average initial length (mm)	E_{Shive} $\pm t_{.05} s_{\bar{x}}$ (mm)	$E_{\text{serum I}}$ $\pm t_{.05} s_{\bar{x}}$ (mm)	$E_{\text{serum II}}$ $\pm t_{.05} s_{\bar{x}}$ (mm)	Relative growth (I_E) $\pm t_{.05} s_{\bar{x}}$ (serum I)	Relative growth (I_E) $\pm t_{.05} s_{\bar{x}}$ (serum II)
14—15/2 1.	50	28	29.8	8.4 ± 1.0	5.5 ± 1.0		100 ± 12	
	50	26	28.6				65 ± 14	
14—15/2 2.	75	30	35.1	9.9 ± 0.8	6.2 ± 0.7		100 ± 8	
	75	30	37.2				63 ± 9	
14—15/2 3.	100	31	40.4	10.9 ± 1.0	6.4 ± 0.8		100 ± 9	
	100	31	41.3				59 ± 9	
14—15/2 4.	125	31	43.8	12.4 ± 1.0	7.6 ± 0.9		100 ± 8	
	125	30	43.0				62 ± 9	
14—15/2 5.	150	32	40.8	11.3 ± 1.1	6.8 ± 0.8		100 ± 10	
	150	31	41.1				60 ± 9	
14—15/2 6.	300	30	46.8	15.0 ± 1.4	9.3 ± 0.6		100 ± 9	
	300	30	45.0				62 ± 7	
21—22/2 7.	100	26	39.8	11.6 ± 1.2	7.6 ± 1.4		100 ± 10	
	100	26	38.2				66 ± 14	
	100	26	39.9			4.4 ± 1.2		38 ± 11
21—22/2 8.	300	32	45.4	15.3 ± 1.1	11.5 ± 0.7		100 ± 7	
	300	33	46.1				75 ± 7	
	300	33	44.9			5.4 ± 0.6		35 ± 5

From the experiments in Table 7 it will, furthermore, be seen that the 95 % confidence intervals are comparatively far larger in the case of the low E_{Shive} values as an expression for the fact that the certainty here is far less than for the higher E_{Shive} values. This, of course, also applies when the growth in an inhibiting or promoting solution has to be expressed as a percentage of E_{Shive} . The greater the uncertainty in the indication of this latter quantity, the greater the uncertainty of the index values derived therefrom.

Table 8, finally, shows the inhibition of *serum* to lupine roots germinated preliminarily in sphagnum of various water percentages. Also here it is noted that the various roots have the same sensitivity to the same serum, irrespective of their culture conditions during preliminary growth.

It may therefore be concluded that lupine roots with different rates of growth have the same sensitivity. The greatest reliability of the measuring results is, however, obtained with roots having a high rate of growth.

Summary

A number of investigations of the growth of *Lupinus albus* roots in nutrient solution and its dependence on varying conditions during germination and growth in a preliminary medium are presented.

It is demonstrated that standard conditions during the preliminary growth of the roots in respect of duration of growth, temperature, moisture content in preliminary growth media, the nature of and degree of washing (degree of purity) of the preliminary media, are all necessary for obtaining reproducible elongation results for roots kept in nutrient solution.

Further, it is demonstrated that various conditions during the preliminary growth (water content in sphagnum, degree of purity of culture medium) exert an exceptionally great after effect on the growth in the nutrient solution. Such after-effects do not appear to have been demonstrated previously.

The elongation of the roots under standard conditions (15°C, 24 hours in Shive's nutrient solution after preliminary growth at 20°C in washed "No-dampoff" sphagnum with 300 % for 60 hours) has been followed by experiments carried out at regular intervals over a period of one whole year.

A number of examples of the sensitivity of lupine roots to various external conditions are given (distilled water, D- and L-chloramphenicol, urea 500 µg./ml. of Shive's solution, human serum 1 ml/100 ml of Shive's solution). It is shown how roots with different rates of growth, originating from preliminary cultivation media of different moisture contents, all have the same sensitivity to the same external influence. The greatest reliability of the results, however, is obtained with roots having a high rate of growth.

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Effect of Saline and Alkaline Salts on the Growth and Internal Components of Selected Vegetable Plants

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Introduction

On the western coast and a portion of the southern coast of Korea large areas of tidal land are frequently found due to the slight degree of inclination of the coastal area and the marked differences between low and high tides. It is estimated that about 160 thousand acres of tidal land are exposed to the air during low tide.

Since the soil of these tidal land and of adjoining land has been subjected to salinization, the soil solution has a high osmotic concentration. Many earlier investigators have noted the osmotic value of the soil solution often acts as a limiting factor in plant growth quite aside from its specific composition (1, 7, 8, 9, 10, 11, 13, 16, 23).

Further, it has been observed with some plants that injury due to salt concentration in the soil solution varies according to the salts used (14, 16). But recently the relation between various salts and internal quality of plants has been a central interest and many workers have investigated this problem (2, 12, 4, 19, 20, 22). According to Kearney and Scofield (15) several kinds of crops may be impaired by a quality of salts too small to interfere seriously with growth of the plants.

Throne and Peterson (21) mentioned that the effects of salts in reducing crop plant growth probably came from one or more of three different sources; first, direct physical effects of the salts in preventing water uptake; second, direct chemical effects of the salts in disturbing the nutrition and metabolism

of plants; and third, the indirect effects of salt in altering soil structure, permeability, an aeration. But it has been recognized that there is often no marked distinction between the effects of various salts and of drouth on the nutrition and metabolism of plants (18).

This series of laboratory experiments with vegetable plants has been designed to measure the effects of various salts on the nutrition of the plants grown in soil cultures under well irrigated conditions.

The study reported here has been undertaken to ascertain the influence of sodium chloride and sodium carbonate which are often found in tidal land, on the growth and on some of the internal components in selected vegetable plants. The radish, cabbage, and lettuce were selected because they are the most common vegetable crops used in Korea.

Experimentation

On April 22, 1956, seeds were planted in pots filled with loam soil brought from the farm yard of College of Education, Seoul National University. The soil had previously been thoroughly mixed, spread out, and dried in the air for several days.

After germination the seedlings were thinned, leaving 10 uniform seedlings in each pot. The plants were watered to maintain the soil moisture in order to keep field capacity. A week after germination the soil was fertilized with 60 kg. per hectare of N, K₂O, P₂O₅ in the form of potassium, calcium nitrate and superphosphate respectively. Two weeks following the fertilization the seedlings of each pot were subjected to a different treatment of salts and after a week following the initiation of salt treatment the plants were used for the first experimentation. The high toxicity of the soil solution arising after the application of salts could be largely eliminated by neutralizing the solution to pH 6.8—7.0 with H₂SO₄. The second experiments in which all procedures were the same as in the first experiment, were performed 3 weeks following first.

A portion of the plant was harvested from each series and details on the appearance of the plant and the fresh and dry weight data were recorded. A week following the different treatment of salts the plants were subjected to chemical determinations.

For the chlorophyll estimation ten samples were removed from many leaves within each series by a cork borer and then the fresh weight was determined before the extraction was made. The method used in the extraction and separation of chlorophyll from the other plant pigments was that of Willstätter and Stoll as modified by Schertz. Determinations of the total chlorophyll content were made by means of Duboscq colorimeter in comparison with Guthrie's chemical standard. In this experiment the chlorophyll was separated from the carotenoid pigments but no attempt was made to separate either chlorophyll α from chlorophyll β or carotene from xanthophyll.

The ascorbic acid was determined with 2,6-dichlorophenolindophenol in acetic acid according to the method of Bessey and King.

For the vitamin C determination, one leaf was taken from one of the plants in each level, and then the leaves were divided longitudinally avoiding the midrib, into halves, and weighed.

Results

Growth: The plants in various treatments of both series were uniform in appearance and there was little variation in growth responses. But a tendency of reducing the growth of the three crop plants with the increasing salt concentration was noticed (Figure 1).

In general the fresh and dry weights of the plants receiving sodium chloride were a little larger in all cases than those of the plants receiving sodium carbonate. But except for a slight pale color apparently due to salts treatment, no visible symptoms of injuries such as chlorosis or dead leaves were noted.

Chlorophyll: The chlorophyll content in plants is given in milligram for fresh weight, and per square centimeter of surface area.

Regardless of the kinds of vegetables plants the content of chlorophyll in plants in which sodium salts were reduced in all cases by increasing the concentrations. But the plants in the sodium carbonate series contained amounts of chlorophyll considerably lower than those of the sodium chloride series (Table 1). It was noticed that the chlorophyll content was reduced in the order raging from high to low, of radish, lettuce and cabbage. Table 1 shows that there was a general tendency toward an inverse relation between the chlorophyll content and level of salt solutions of the two kinds of salts. The differences in chlorophyll reduction, however, were comparatively small.

Since the amounts of chlorophyll present in both series were proportional to the amount of salts supplied in the soil culture, it is probable that the level of salt concentration, rather than the other factors within the soil, was the determinant factor for the reduction of chlorophyll content.

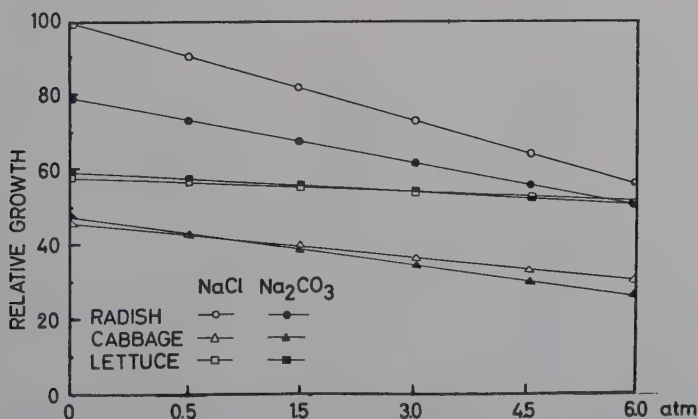


Figure 1. Growth response of radish, cabbage, and lettuce to different treatments of sodium chloride and sodium carbonate in soil culture.

Table 1. *Effect of concentrations of two salts in soil culture on the content of chlorophyll of three vegetable plants.*

Concentration (atm.)	Radish			
	NaCl		Na ₂ CO ₃	
	mg./100 gm. fresh wet.	mg./100 sq. cm.	mg./100 gm. fresh wet.	mg./100 sq. cm.
Control	100.2	3.84	100.2	3.84
0.5	90.0	3.47	87.6	3.24
1.5	82.6	3.18	85.2	3.15
1st 3.0	86.4	3.28	80.0	3.08
4.5	74.4	2.98	73.0	2.96
6.0	75.6	2.84	70.4	2.98
Control	89.6	3.23	89.6	3.45
0.5	85.0	3.23	73.6	2.93
1.5	80.2	2.93	72.8	2.84
2nd 3.0	80.4	3.10	75.6	2.91
4.5	72.8	2.95	70.6	2.83
6.0	61.8	2.72	68.2	2.83
Cabbage				
Control	91.2	3.28	91.2	3.28
0.5	80.6	2.86	81.2	3.00
1.5	70.0	2.49	70.4	2.60
1st 3.0	73.2	2.56	69.0	2.55
4.5	64.0	2.37	61.0	2.32
6.0	62.8	2.42	61.0	2.32
Control	68.0	2.36	68.0	2.62
0.5	58.8	2.29	58.0	2.32
1.5	52.0	2.16	49.8	1.94
2nd 3.0	50.0	1.95	49.8	1.97
4.5	46.6	1.72	43.0	1.72
6.0	42.2	1.79	34.0	1.34
Lettuce				
Control	74.6	2.46	74.6	2.46
0.5	71.0	2.44	68.8	2.41
1.5	72.4	2.39	68.0	2.27
1st 3.0	70.8	2.31	63.2	2.15
4.5	64.2	2.21	51.2	1.82
6.0	51.6	1.78	43.4	1.52
Control	68.2	—	68.2	—
0.5	70.2	—	64.4	—
1.5	71.0	—	63.2	—
2nd 3.0	72.2	—	58.8	—
4.5	55.4	—	46.4	—
6.0	50.2	—	40.0	—

P-values for analysis of variance are: Salts=0.69, 1.37, 1.85, Concentration=4.24**, 23.84**, 2.66*, and Periods=4.86*, 175.28** 0.58 in radish, cabbage, and lettuce respectively. — * significant at the 5 % level, ** at the 1 % level.

Table 2. *Effect of concentrations of two salts in soil culture on the content of carotene of three vegetable plants.*

Concentration (atm.)	Radish			
	NaC 1		Na ₂ CO ₃	
	mg./100 gm. fresh wet.	mg./100 sq. cm.	mg./100 gm. fresh wet.	mg./100 sq. cm.
Control	45.2	1.70	45.2	1.70
0.5	43.2	1.66	40.2	1.54
1.5	42.0	1.67	39.2	1.45
1st 3.0	44.0	1.62	40.0	1.49
4.5	35.4	1.42	35.2	1.43
6.0	30.4	1.22	32.4	1.33
Control	44.2	—	44.2	—
0.5	43.4	—	40.0	—
1.5	41.2	—	40.0	—
2nd 3.0	40.0	—	40.0	—
4.5	31.2	—	30.4	—
6.0	32.8	—	28.8	—
Cabbage				
Control	40.6	1.46	40.6	1.46
0.5	39.2	1.38	40.2	1.33
1.5	38.8	1.37	39.2	1.31
1st 3.0	35.6	1.26	36.4	1.30
4.5	30.4	1.12	30.4	1.19
6.0	28.4	1.09	26.6	0.97
Control	41.4	—	41.4	—
0.5	36.0	—	36.0	—
1.5	34.4	—	35.4	—
2nd 3.0	33.0	—	35.0	—
4.5	27.0	—	31.2	—
6.0	27.6	—	25.6	—
Lettuce				
Control	36.0	1.19	36.0	1.19
0.5	32.2	1.06	30.0	0.99
1.5	30.2	1.05	27.8	0.95
1st 3.0	30.4	1.00	25.4	0.90
4.5	28.8	0.99	20.2	0.72
6.0	24.4	0.84	19.8	0.69
Control	30.2	—	30.2	—
0.5	26.6	—	31.4	—
1.5	26.0	—	30.8	—
2nd 3.0	25.0	—	29.8	—
4.5	24.0	—	23.2	—
6.0	23.6	—	21.0	—

P-values for analysis of variance are: Salts=1.56, 0.40, 0.39, Concentration=42.08**, 33.84**, 2.73*, Periods=4.01, 0.19, 1.05 in radish, cabbage, and lettuce respectively. * and ** as in Table 1.

Table 3. *Effect of concentrations of two salts in soil culture on the content of ascorbic acid (in mg./100 g. fresh weight) of two vegetable plants.*

Concentration (atm.)	Radish		Cabbage	
	NaCl 1	Na ₂ CO ₃	NaCl 1	Na ₂ CO ₃
Control	145.1	145.1	105.0	105.0
0.5	136.9	139.8	104.8	103.7
1.5	119.7	131.6	86.6	103.1
1st 3.0	112.3	123.9	69.1	89.5
4.5	112.0	122.7	67.8	84.1
6.0	102.5	103.8	64.2	82.6
Control	172.6	172.6	124.7	124.7
0.5	170.6	162.5	122.0	116.9
1.5	159.8	152.3	104.1	115.5
2nd 3.0	148.4	140.2	102.6	114.4
4.5	127.1	137.8	101.2	112.5
6.0	126.3	141.3	96.7	110.2

P-values for analysis of variance are: Salts=0.39, 9.18**, Concentration=5.14**, 8.37**, Periods=31.21**, 54.87** in radish and cabbage respectively. ** as in Table 1.

Carotene: The carotene content of the plants grown under various treatments varied markedly. As the concentration of salts added were increased there was a reduction of carotene content.

Carotene was higher in the plants of the sodium chloride series than in the plants of the sodium carbonate series in all vegetables tested. Table 2 shows that the carotene content of the leaves of different ages had nearly the same values, although the mature leaves were somewhat lower in carotene than the young leaves.

Table 2 also shows that there was always considerably more chlorophyll present than carotene. Deleano (5) and other investigators found that changes in the content of carotene in the willow and soy bean advances parallel with the changes in the content of chlorophyll, and consequently the ratio of yellow and green pigments remain constant. According to Beck and Redman (3) the content of chlorophyll on an average was 36.5 times greater than carotene. In this study the result shows no similar relationship. In fact little difference was noted between content of chlorophyll and carotene. The comparisons did show, however, that the distribution of carotene paralleled with that of the chlorophyll.

Ascorbic acid: The ascorbic acid content of plants in this experiment was also reduced with the increasing salts concentration. It was surprising, therefore, that ascorbic acid values were considerably greater in the plants of the sodium carbonate series than in the plants of the sodium chloride series. This

difference was highly significant, with the exception of lettuce in which there was no experimentation with the values of ascorbic acid (Table 3).

Keller and Minot found that in ten samples the ascorbic acid content of fresh turnip greens varied from 75—160 mg. per 100 mg. fresh weight. The results of this experiment showed the ascorbic acid values to be approximately the same. It was also noticed that the ascorbic acid content increased with the time lapse. That is, the ascorbic acid content was higher in the leaves of plants in the second experiments than those used in the first experiments.

Discussion

The foregoing data show that little variations of concentrations of the sodium chloride and sodium carbonate added in soil tended to reduce the amounts of chlorophyll, carotene, and vitamin C as well as the growth of three vegetable plants. The inhibition of growth, however, was not so marked as the changes in the internal components. These changes have a correlation with variations in different levels of salts supply. Although there no significant differences were evident, the growth of the plants in sodium carbonate series was less than that of plants in the sodium chloride series. This differences was due to the kind of salts and not the osmotic levels. These experiments show that the growth reduction is probably due to the chemical effects of the sodium carbonate in disturbing the nutrition and metabolism of plants. The experiments further show that the rate of growth advances in remarkably close parallel relationship with changes in the content of chlorophyll and carotenoid pigments in the three vegetable plants. Such relationship would seem highly logical since as mentioned by Beck and Redman (3), all energy involved in growth processes enters the plants through the catalytic activity of chlorophyll. Carotene serves directly or indirectly as a growth promoter.

In order to evaluate quantitatively the interrelationships among the internal components, these data obtained in this experiments were reduced statistically by analysis of variance and regression to obtain the summary values.

The statistics show that the variations of chlorophyll in three plants were directly related to the level of sodium chloride and sodium carbonate to a remarkably high degree, although no significant differences are evident between the two salts. Maiwald (17) observed that the content of chlorophyll was reduced under the influence of potassium salts in potatoes. Dhein (6) also has observed an actual reduction of the amount of chlorophyll only with large doses of KCl. Wadleigh and Brown (22) have shown that the chlorosis caused by chlorophyll deficiency in bean plants was induced by adding the NaHCO_3 to a nutrient solution. It was also noticed that the relation of chloro-

phyll content in the three crop plants to the concentration of the two salts became more pronounced with lapsed time. According to Sideris and Young (19) the content of chlorophyll in the leaves of *Ananas comosus* was reduced progressively toward the end of the growing period. This is in agreement with the belief that conditions reducing the development of chlorophyll are concomitant with the senility of leaves.

Carotene content in the leaves of the three plants treated by two salts varies in parallel with the chlorophyll. The salts treatments had a direct effect on caroten content in the three plants. There were slight differences in the amounts of carotene in the leaves of different ages. However, these differences were not as significant as those of the chlorophyll. Bernstein *et al.* (4) recognized a similar condition as in this experiment when they found, that although the leaves of different ages had nearly the same carotene concentration, the concentration in the oldest leaves was somewhat lower than in the other age groups. Deleano and Dick (5) also observed that there was no difference in the content of carotene between green and yellow green leaves of willow. This evidence may indicate that there would be a high degree of concomitance between growth rate and the contents of chlorophyll and carotene, since the status of chlorophyll and carotene, may be a merely a primary factor conditioning growth of these crop plants.

Cabbage grown in soil showed indication that salts treatment influenced ascorbic acid content to a high degree of significance. However in the radish no significant differences were evident, in spite of the fact that the salts treatment employed had an effect upon the growth of the plants.

An analysis of variance of the data on the ascorbic acid of the radish and cabbage, when these were treated by two salts, showed significant differences between the two salts. Contrasted with the other two components, *i.e.*, chlorophyll and carotene, ascorbic acid values were higher in the sodium carbonate series than in the sodium chloride series. A progressive increase in the amounts of ascorbic acid with the time of growth was also highly significant. Sideris and Young (19) stated in their investigations that ascorbic acid content were not directly proportional to that of chlorophyll in the leaves of *Ananas comosus*, although ascorbic acid was almost limited to the chlorophyllous section of the leaves. Bernstein, Hamner and Parks (4) stated that average ascorbic acid values differed greatly during different seasons. The differences in the amounts of ascorbic acid noticed between the first and second experiments may be explained by the fact that ascorbic acid is produced in greater amounts in leaves of great vigor than in immature leaves. The phase of distribution of ascorbic acid in leaves of radish and cabbage suggest that certain phases of metabolism rather than amounts of chlorophyll in these tissues were responsible for their ascorbic acid content.

Summary

1. The effects of saline and alkaline salts on the growth as well as the internal components of the leaves of ordinary vegetable plants under the soil culture was investigated.

2. These results have indicated that small variation in the relative concentrations of the two salts in the soil has shown a marked influence on the internal components of three plants.

3. The reduction of growth was associated with increasing intensity of salts, although no significant differences was evident.

4. Chlorophyll and carotene content in the leaves of three plants were depressed with increasing concentration of two types of salts, but the different effect of the two salts was not significant.

5. It is noticed that there was a high degree of concomitance between growth rate and the contents of chlorophyll and carotene.

6. In contrast the ascorbic acid content in the leaves of cabbage and radish treated with two salts increased in proportion to the degree of the concentrations of the two salts. The difference was highly significant of the two salts in cabbage.

7. Chlorophyll and ascorbic acid values differed greatly during the two experiments and the difference was significant.

8. Carotene content varies with chlorophyll although the ratio of chlorophyll to carotene was not so much high as the results obtained by Beck and Redman (3).

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Effects of Gibberellic Acid on the Growth and Flowering of Telephone Peas

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Introduction

The physiological effects of the gibberellins on plants have received much attention, and the subject has recently been reviewed by Stowe and Yamaki (1957). The effect first noted was primarily stimulation of vegetative growth, but evidence has accumulated that the flowering behavior of various plants is influenced by applied gibberellin.

In their study of the effects of gibberellin on the flowering of beans, tomatoes, and lettuce, Wittwer *et al.* (1957) found that earlier flowering resulted from an acceleration of vegetative growth but that there was no reduction in internode number. Marth *et al.* (1956) found no evidence that gibberellic acid could induce the formation of flower primordia in the forty-nine species that they studied.

Lang, however, showed that gibberellin could replace the cold requirement for flowering of biennial *Hyoscyamus* (1956 a, 1956 b) and the long-day requirement in an annual variety of *Hyoscyamus* and in other long-day plants (1956 c, 1957). Bünsow and Harder (1956 a) found that gibberellin could replace the long-day portion of the photoinductive requirement of two species of *Bryophyllum* and affect the flowering of various other species (1956 b, 1956 c, 1957). Harrington *et al.* (1957) reported that gibberellic acid induced non-vernalized endive plants to flower, and Wittwer and Bukovac (1957) showed that gibberellin could be substituted for the cold or long-day requirements for flowering in a number of species. Gibberellins thus appar-

ently can take the place of the long-day and cold requirements necessary for flowering in numerous species, but it has not been found to replace short-day requirements.

The vegetative responses of peas to gibberellins have been studied rather intensively by Brian and Hemming (1955), and these authors have noted the greater response of dwarf than tall varieties to gibberellin. The present study is concerned with the effects of gibberellic acid (GA) on the growth and flowering of the dwarf variety of the Telephone pea, *Pisum sativum* L., and to a small extent with the tall variety of the Telephone pea.

Materials and Methods

Dwarf and tall varieties of the Telephone pea were used in this study. The seeds were planted in vermiculite in flats, and six days later groups of twenty uniform seedlings were transplanted into flats of soil. The plants were supplied with nutrient solution at intervals of seven to ten days during the growing periods and were given supplemental light in the evening to provide sixteen hours of light per day.

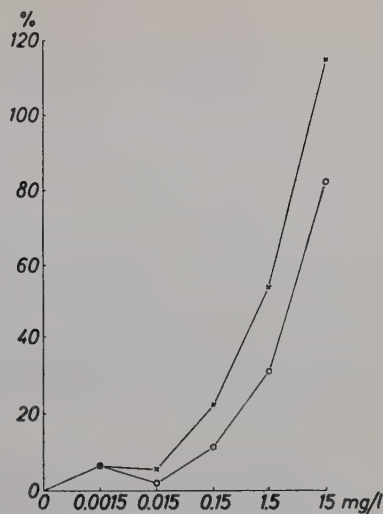
Ten days after planting, a series of flats of dwarf seedlings was sprayed until the plants were thoroughly wetted with solutions of the potassium salt of gibberellic acid (GA), each flat being sprayed with one of the following concentrations: 0, 0.0015, 0.015, 0.15, 1.5, and 15.0 mg./l. Stock solutions of GA were prepared by dissolving the crystalline material in a small amount of ethanol and diluting this solution with distilled water to the desired concentrations. A small amount of Tween 20 was added to each solution as a wetting agent. A second series of flats with twenty plants in each was sprayed in the same manner when the seedlings were twenty days old. The plants in both series were harvested sixty-four days after planting, at which time data on the vegetative and flowering conditions of the plants were taken.

A group of twenty tall Telephone peas was sprayed with 15 mg./l. GA sixteen days after planting, and another group was left untreated.

Results

Figure 1 shows the percentage increases in height in the two series of dwarf plants over that of the controls sixty-four days after planting, at which time the plants all were flowering. The growth responses are quite parallel, but they are greater in the case of the plants sprayed at twenty days after planting than after ten days. This difference in the amount of vegetative response to GA may be in part explained by the fact that the larger twenty-day-old plants received more of the spray by foliar absorption and thus were affected more than the younger plants with less leaf surface. Approximately

Figure 1. *Effects of single sprays of various concentrations of gibberellic acid on growth in height of dwarf Telephone peas. One series of plants was sprayed at 10 days (○—○) after planting and the other at 20 days (×—×). Averages of 15 to 18 plants in each group are presented. On the abscissa gibberellic acid mg./l., on the ordinate increase in height over controls.*



4 ml. of solution per plant was used to wet the younger plants uniformly, while approximately 5 ml. was used for each of the older ones.

The increase in height of the sprayed plants was accompanied by the commonly observed chlorosis of the leaves and general spindly appearance of the stems. Fresh weights and dry weights taken at harvesting show an increase in those values with concentrations of GA in both series of plants. This amounted to an increase in fresh weight of 70.9 % over the controls in the 10-day plants and 36.5 % in the 20-day plants at the highest concentration. Dry weight was increased 107.9 % over the controls in the 10-day plants and 86.9 % in the 20-day plants at this same concentration. These figures may be contrasted with the height increases, 82.7 % for the 10-day plants and 115.6 % for the 20-day plants, which show that even though the earlier sprays produced heavier plants, these plants were not as tall as those sprayed later. At the three lowest concentrations the dry weights of the plants sprayed at 10 days were smaller than the weights of those sprayed at 20 days, however.

Measurements of petiole lengths and stipule and leaflet widths at the tenth node at harvesting gave little indication that the growth of these structures was consistently affected by the GA, although in some of the concentrations there were increases with the various sprays, particularly when they were applied at the earlier date.

Data on the effect of the different GA concentrations on the number of nodes to the first flower in the dwarf pea are presented in Figure 2. The number of nodes to flower in the tall variety of Telephone pea was unchanged

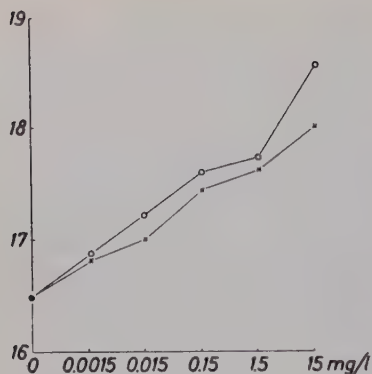


Figure 2. Effects of single sprays of various concentrations of gibberellic acid on the number of nodes to flower in dwarf Telephone peas. One series of plants was sprayed at 10 days (○—○) after planting and the other at 20 days (×—×). Averages of 15 to 18 plants in each group are presented. On the abscissa gibberellic acid mg./l., on the ordinate number of nodes to flower.

by 15 mg./l. GA, but the dwarf variety, on the other hand, responded to increasing concentrations of GA with increasing numbers of nodes to the first flower in both series of plants. The plants sprayed at ten days were slightly more affected in this respect than were the ones sprayed at twenty days of age.

Discussion

Gibberellic acid is well recognized as a stimulator of vegetative growth in a great variety of plants. In addition, it has been shown by various workers to hasten flowering, although it apparently does so indirectly, since the authors found that the number of internodes in the treated plants is unaffected. Dwarf Telephone peas show the remarkable effects of GA on vegetative growth. In the data presented here the vegetative response in these plants was greater when the GA was applied at 20 days of age than at 10 days so far as the plant height is concerned. The opposite is true of the effects on the fresh weights and dry weights with the two higher concentrations, however.

The reproductive response of the dwarf Telephone pea is interesting in that increasing concentrations of GA, in the range tested, delay flowering morphologically, *i.e.*, cause the first flowers to form at higher nodes than in the untreated plants. Nevertheless, the controls and the treated plants all bloomed at about the same time, because the increased rate of vegetative growth of the treated plants hastened the appearance of the flowers chronologically. The effect on flowering of the single GA sprays of the different concentrations was greater when the sprays were applied earlier in the life history, whereas the effects on heights of the plants were greater with the later application. GA appears to be involved in the flowering process in the dwarf

pea in a manner opposite to the cold-replacing and long-day-replacing effects previously described in that flowering is morphologically delayed although not chronologically.

Conclusion

1. Single sprays of increasing concentrations of gibberellic acid (GA) in the range of 0.0015 to 15.0 mg./l. have increasing stimulatory effects on the stem elongation of dwarf Telephone peas. Height increases at all concentrations were greater in plants treated at 20 days than in those treated at 10 days of age. In contrast, fresh and dry weight increases were greater in plants sprayed at 10 days than at 20 days at the two highest concentrations but not at the lower ones.
2. The time required to flower in the dwarf Telephone pea was not affected by GA treatment. However, increasing concentrations of GA increased the number of nodes to the first flower to a maximum increase of two nodes at the highest concentration. Fifteen mg./l. had no effect on flowering in the tall variety. A spray of a given concentration applied at 20 days of age was less effective than one applied at 10 days in changing the number of nodes to flower in a group of plants.

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Effect of Low Concentrations of Ozone on the Enzymes Catalase, Peroxidase, Papain and Urease

By

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Ozone gas at low concentrations is toxic to both plants (Middleton, 1955) and animals (Stokinger, 1954). Ozone at a concentration of 1 part per million (by volume) or less affects the photosynthesis and respiration of *Lemna minor* (Erickson and Wedding, 1956) and the respiration of lemon fruit and bean leaves (Todd, 1956, 1957). It is possible that some of these effects are produced by direct action of the ozone on some of the enzymes within the cell. Although many enzymes are known to be susceptible to damage by oxidizing agents such as hydrogen peroxide (Sumner and Somers, 1947; Barron, *et al*, 1952), the effect of ozone, which is an extremely active oxidizing agent, has not been investigated to any extent. It has been shown that ozone is capable of inactivating crystalline catalase, but rather high concentrations of ozone were used (Giese and Christensen, 1954).

The present work concerns the action of ozone on a few representative enzymes, and an attempt has been made to estimate the actual quantity of ozone required for inactivation.

Methods and Materials

Enzyme Preparations

Catalase. Beef liver catalase was prepared by the method of Sumner and Somers (1947). The dialyzed catalase preparation had a Kat. f. of 1035. The preparation

¹ Paper No. 1015, University of California Citrus Experiment Station, Riverside, California.

Table 1. *Physical characteristics used to calculate active enzyme present.*

Crystalline Enzyme	Molecular weight	Units of activity per gm. of crystalline enzyme	Reference
Urease.....	473,000	133,000	(Sumner, 1951)
Catalase (beef liver)	248,000	30,000	(Sumner and Somers, 1947)
Peroxidase	44,000	900	(" " " ")
Papain	30,000	4,030	(Balls and Lineweaver, 1939)

used in the experiments contained 0.045 gm. of enzyme per liter in 0.05 *M* phosphate buffer pH 7.0.

Peroxidase. This enzyme was prepared from turnips by blending in a Waring Blendor with water and precipitating with ammonium sulfate. The precipitate was dialyzed, and the enzyme activity measured by the method of Siegel (1953). The enzyme gave a P.Z. of 2.99, and the preparation used for treatment had a dry weight of 0.30 gm. per liter.

Papain. A commercial preparation of this enzyme (from Nutritional Biochemicals) was used, and the coagulation of milk was used as the assay (Balls and Hoover, 1937). One ml. of the enzyme solution (containing 1.18 gm. per liter) had an activity of 0.143 milk clot units at 30°C.

Urease. This enzyme (from Nutritional Biochemicals) was suspended in 0.067 *M* pH 7.0 phosphate buffer and filtered. The final solution contained 0.12 gm. per liter, and the activity was 117 units per gm. as measured by the method of Sumner and Hand (1928).

Ozone Production

The ozone was produced by a silent discharge tube through which commercial-grade oxygen dried with Drierite was passed. This gas stream was diluted with activated charcoal-filtered air to produce the desired concentrations of ozone. For the urease studies the ozone was produced by passing the oxygen over a Westinghouse Model 794H "odorout" tube in order to obtain lower concentrations than were obtainable by the discharge tube. The voltage supplied to both ozonizers was regulated at 115 volts (± 5 per cent) to reduce fluctuations in the ozone output. The diluted gas stream containing the ozone was divided in half, and while the enzyme was being treated with part of the gas stream in a midjet impinger, the other half of the stream was impinged through 2 per cent buffered potassium iodide solution (0.1 *M* phosphate buffer, pH 7.0). The iodine released from the potassium iodide by the ozone was read in a Beckman Model DU spectrophotometer at 360 m μ or was titrated with standard potassium thiosulfate. In some of the experiments the gas stream, after impingement through the enzyme solution, was analyzed for its ozone content.

One-tenth to 1.5 cu. ft. of air containing 0.5–10 ppm ozone (by volume) was passed through an enzyme solution. In some of the enzyme studies the concentration of ozone remained constant and the total volume of gas used was changed; in other studies the concentration of ozone was changed and the volume of gas was kept

constant. Both methods gave similar results. All inhibition results have been corrected for the inhibition obtained with oxygen-air mixtures containing no ozone.

In order to compare the effects of ozone on these several enzymes, the activities are expressed in terms of active enzyme molecules present. The actual weight of pure enzyme that might be present in these preparations was calculated using the activity measurements and molecular weights given in Table 1. The number of moles of enzyme could then be calculated from the molecular weight, assuming that the crystalline enzyme used by other workers for their determinations was 100 per cent active.

Results

The effect of ozone on the enzymes catalase, peroxidase, papain and urease is shown in Figure 1. All the enzyme inactivation curves presented are results of tests made on a single enzyme preparation on a given day. There was some variability with certain enzymes from day to day, owing to slight differences in the enzyme preparations. While the enzymes peroxidase and catalase are capable of decomposing peroxides which are active oxidizing agents, it is evident that ozone can cause considerable enzyme inhibition. Catalase is, of course, somewhat inactivated by its own substrate, hydrogen peroxide (Sumner and Somers, 1947). Peroxidase was partially protected from inactivation by ozone when hydroquinone was present during the impingement of the enzyme with the ozone gas. A concentration of 1×10^{-3} *M* hydroquinone reduced the extent of inactivation by 40 to 60 per cent when amounts of ozone up to 0.5 μ mole of ozone was introduced per ml. of enzyme. It seems likely that the ozone was reacting preferentially with the hydroquinone, when it was present, rather than with the peroxidase. Papain appeared to be more reactive to ozone than the other enzymes tested. There was a possibility that papain might be more sensitive to ozone when treated in the cysteine-activated state. However, the order of addition of cysteine and ozone to the papain did not change the amount of inhibition of the enzyme. This suggests that the activation state of the enzyme does not change the susceptibility to ozone. The amounts of ozone required for 50 per cent inhibition of the four enzymes are given in Table 2. The amount of ozone required on a dry-weight basis of the particular enzyme preparation is given, as well as the methods used in calculating the amount of pure enzyme present.

The measurement of the ozone introduced into the solution did not give any indication of the amount of retention of the ozone by the enzyme solution. Thus the amount of ozone required for a given amount of inactivation could be considerably less than is indicated in Figure 1 and Table 2. Experiments were performed with urease and papain in which the amount of ozone escaping from the enzyme solution was determined. Since the total ozone introduced was known, the amount absorbed by the enzyme solutions

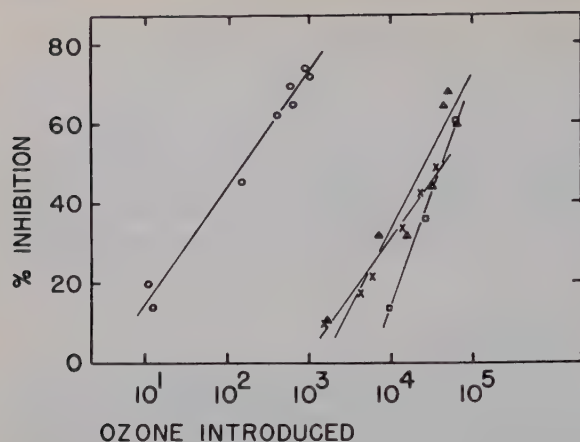


Figure 1. Inhibition of enzymes by ozone (moles of ozone per mole of enzyme): \circ , papain; Δ , urease; \times , peroxidase; and \square , catalase.

was calculated. The data obtained in this manner for urease and papain, showing the relation of inhibition to the ozone actually absorbed, are given in Figure 2. The amounts of oxidant required to produce a 50 per cent inactivation of urease and papain are given in Table 3 on the basis of both a per gram dry weight of enzyme and per mole of active enzyme. The urease solution removed an average of 23 per cent of the ozone introduced, while the papain solution removed an average of 61 per cent of the gas introduced.

Table 2. Amounts of ozone required for 50 per cent inhibition of enzymes.¹

Enzyme	Ozone in solution, millimoles per liter ²	Enzyme concentration gm. per liter ³	Ozone required, millimoles per gm. dry weight of enzyme prep. ⁴	Active enzyme, moles per liter ⁵	Moles ozone per mole active enzyme ⁶
	(1)	(2)	(3)	(4)	(5)
Urease.....	0.05	0.12	0.416	1.65×10^{-9}	30,300
Catalase	0.28	0.045	6.23	6.2×10^{-9}	45,160
Peroxidase ...	0.80	0.030	27.10	2.2×10^{-8}	36,400
Papain	0.28	1.18	0.241	1.2×10^{-6}	233

¹ On basis of total ozone introduced into solution (see Table 3 for actual absorption measurements).

² Ozone concentration obtained from plot of ozone required for 50 per cent inactivation.

³ Dry weight of particular enzyme preparation used.

⁴ Column 1

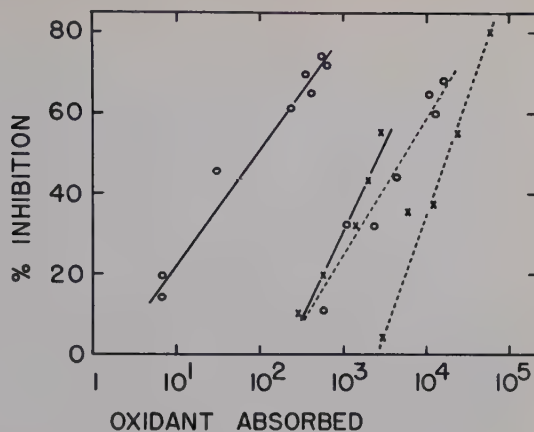
Column 2

⁵ Calculated from data on crystalline enzymes.

⁶ Column 1

Column 4

Figure 2. Inhibition of the enzymes papain and urease by ozone and hydrogen peroxide (moles of oxidant per mole of enzyme): \circ , ozone; \times , hydrogen peroxide; ----, urease, and — papain.



One factor responsible for this difference could be that the papain solution was more concentrated than the urease solution and was therefore more efficient in removing the ozone. The papain may also contain structural groups that combine with the ozone more readily than groups contained in urease.

Several tests were made with the enzyme papain to determine whether any residual free ozone was left in the enzyme solution. After impingement of the enzyme with ozone there was nothing left in the enzyme solution that would produce iodine from potassium iodide solution. Thus all the ozone was destroyed by, or combined with, the enzyme solution.

The action of hydrogen peroxide on urease and papain might be expected to be similar to that of ozone. Figure 2 shows, however, that inhibition of urease and papain by hydrogen peroxide was much less than that caused by ozone. For a 50 per cent inhibition of papain, 30 times as much hydrogen peroxide was required as for ozone (Table 3). With urease, hydrogen peroxide was only one-third as effective as ozone in the inhibitory action on the enzyme.

Table 3. A comparison of the effectiveness of H_2O_2 and ozone on the inhibition of urease and papain. Concentration of oxidant required for 50 per cent inhibition.¹

Enzyme	Ozone, μ moles per gm. dry wt. enzyme prep.	H_2O_2 , μ moles per gm. dry wt. enzyme prep.	Ozone, moles per mole of active enzyme	H_2O_2 , moles per mole of active enzyme
Urease	83.4	117.0	5,500	18,000
Papain	145.0	977.0	92	2,800

¹ Ozone actually absorbed by the solution.

Discussion

Barron *et al.* (1952) and Hellerman *et al.* (1933) found that 10^{-3} M hydrogen peroxide had no effect on urease, whereas the present urease preparation was inhibited 50 per cent by 8×10^{-6} M hydrogen peroxide. They also found that 10^{-3} M hydrogen peroxide inhibited their papain preparation 40 per cent whereas the present enzyme preparation was inhibited 40 per cent by 2.1×10^{-3} M. Since those authors do not give the enzyme concentrations used, it is not actually possible to compare the results directly, although the results obtained for papain agree quite well. Barron indicates that urease probably contains —SH groups of the “sluggish” type, whereas papain —SH groups are more reactive.

The fact that low concentrations of ozone are toxic to plant leaves suggests the possibility of direct inhibition of plant enzymes by ozone. The amount of ozone required to produce visible damage in leaves has been determined (Todd, unpublished results). It was found that a Pinto bean leaf which had absorbed 40 µg. of ozone per gm. fresh weight was damaged to the extent of 30 per cent of the leaf surface. A comparison between this quantity and the amount of ozone shown above to be required for the inactivation of certain enzymes can be made. A considerable amount of the ozone required for leaf damage may have decomposed on the leaf surfaces and had no contact with living cells. The normal amount of protein in these leaves is between 3.5–4.0 per cent of the fresh weight. Thus the amount of ozone required for leaf damage corresponds to 20–25 µmoles ozone per gm. of protein.

The amounts of ozone required for 50 per cent inhibition of urease and papain for the preparation used are 83 and 145 µmoles ozone per gm. dry weight (essentially all protein), respectively (Table 3). This is considerably more ozone than was required to produce leaf damage. This comparison is based on many assumptions, since it is impossible to determine the amounts of ozone that would be removed by unrelated reactions on the leaf surface or in the cell and by reaction with contaminants in the enzyme preparations. These factors would reduce the amount of ozone available for reaction with the enzymes and alter the comparative values obtained above. If, for example, it is assumed that the ozone was absorbed only by the damaged portion of the leaf (about 30 per cent of the surface), the differences obtained would be reduced by a factor of 3.

It is possible that some of the cellular enzymes are much more sensitive to ozone than the most sensitive of those tested. It would appear, however, that with enzymes similar in structure or function to those tested, the direct action of ozone on enzymes would not be a significant factor in leaf damage. If the ozone does not come in contact with enzymes within the cell, then it

is possible that it reacts with the cell surface. This explanation is compatible with the suggestions of other workers (Giese and Christensen, 1954; Erickson and Wedding, 1956), that the mechanism of ozone toxicity is related to changes in the cell membrane.

Summary

1. The enzymes catalase, peroxidase, papain and urease were treated *in vitro* with low concentrations of ozone gas. Wide variations were found in the sensitivity of the enzymes to the inhibitory action of the gas. Papain showed the greatest sensitivity; the rest required a much greater amount of ozone for inactivation.

2. Comparisons of ozone and hydrogen peroxide as inhibitors of papain and urease showed ozone to be 30 times as effective as hydrogen peroxide on papain and 3 times as effective on urease.

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The Stimulation of Leaf Respiration by Respiratory Inhibitors

By

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Introduction

Leaf tissue provided much of the material for the pioneer work on plant respiration. In recent years it has continually been used in studies on the nature of the respiratory terminal oxidase. (Marsh and Goddard, 1939; Merry and Goddard, 1941; Bonner and Wildman, 1946; Bonner, 1948; Stenlid, 1949; Webster, 1952; Daly, 1954; Daly and Brown, 1954). Such studies generally necessitate the use of heavy metal inhibitors which are known to inactivate specific metal enzyme systems and results so obtained provide a useful pointer to the nature of the respiratory mechanism, the more especially if the degree of inhibition is well-marked and substantial. However, it is not uncommon to find in recent papers dealing with the effect of inhibitors on respiration, records of a stimulatory action on respiration of the so-called respiratory poisons. For instance respiratory stimulations have been reported from the use of carbon monoxide with carrot leaves (Marsh and Goddard, 1939) and with Elodea, Lemna, tobacco and plum leaves (Daly, 1954; Daly and Brown, 1954). Cyanide also stimulated the respiration of carrot leaves (Marsh and Goddard, 1939). Merry and Goddard (1941) obtained a stimulation in barley seedlings in the presence of azide as did Stenlid (1949) with carrot leaves and MacNulty and Newman (1957) stimulated the respiration of bush bean and gladioli leaves with hydrogen fluoride. A feature common to this diversity of material is that the stimulations were all observed in mature tissue. Marsh and Goddard (1939) have already drawn attention to

the fact that young and old leaf tissue differ markedly in their sensitivity to inhibitors and the present investigation was undertaken to obtain more information on the stimulatory effect of several inhibitors on ageing leaves.

Materials and Methods

Leaves from mustard (*Sinapis alba*), tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tabacum*), belladonna (*Atropa belladonna*), avocado pear (*Persea americana*), runner bean (*Phaseolus multiflorus*), maize (*Zea mays*), spinach (*Beta vulgaris*) as well as dwarf shoots of larch (*Larix leptolepis*) and cedar (*Cedrus atlantica* and *C. libani*), all obtained from the Institute greenhouse and gardens, were used in these respiration studies carried out with a conventional Warburg micro-respirometer in the dark at 25°C. Disks were struck from the leaves using a sharp cork borer of 1 cm. diameter. Small leaves were sectioned into halves or comparable pieces and such pieces or disks from the same leaf were used in control and inhibitor experiments. Before use the tissue was vacuum infiltrated with Sorensen's $1/15$ M phosphate buffer (pH 6). Infiltration was easily achieved by evacuation with a water pump. After light blotting the material was transferred to vessels containing a total volume of 2.5 ml. experimental fluid. Each vessel generally contained 10 leaf disks the average dry weight of which varied between 15—30 mg. according to the species. Readings were taken over a 3 hour period. Cyanide experiments were conducted according to Robbie (1948) and carbon monoxide experiments by the evacuation procedure described by Umbreit, Burris and Stauffer (1951).

Results

1. *Effect of inhibitors on respiration of successive leaves.* The respiration rate of successive leaves of a mature mustard plant which had just formed apical flower buds is shown in Figure 1. The leaves are numbered from the apex downwards. The respiratory pattern follows the customary downward curve. In Figure 2 is shown the percentage inhibition or stimulation obtained from the effect of different inhibitors on successive leaves of somewhat younger and smaller mustard plants bearing not more than 7 or 8 usable leaves. As the Warburg apparatus could accommodate only 6 replicates, the lowermost leaves were rejected. 2×10^{-4} M KCN, 95 % CO, 1×10^{-4} M 2,4-DNP, and 5×10^{-4} M NaN₃ were all used at pH 6. The four inhibitors under the same experimental conditions, appear to affect the tissue in a similar manner. Some further examples of the effect of cyanide and azide on the respiration of leaves of varying age from *Zea mays*, *Atropa belladonna* and *Phaseolus multiflorus* are shown in Table 1.

The response to various inhibitors of the youngest fully expanded and the oldest green leaf on a series of uniformly grown tomato plants is shown in Table 2. If the inhibition values for old leaves are plotted on the abscissa and the inhibition values for young leaves on the ordinate, the points indicated in Figure 3 are obtained. These points clearly fit a straight line.

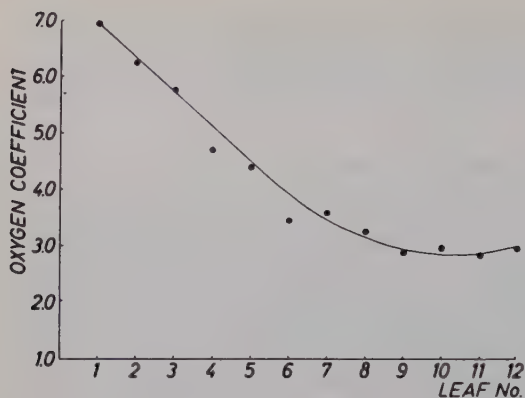


Figure 1. *Respiration rate ($\mu\text{l}/\text{mg. d.wt.}$ and hr.) of Successive Leaves of Mustard Plants.*

2. *Effect of Potassium Cyanide on respiration of ageing leaves.* To answer objections that sampling all the leaves on a plant at any one time does not constitute a true age series accurately reflecting the real nature of the changes occurring in any given leaf as a result of senescent processes, it was decided to sample the same leaf at different times. A tobacco plant is well suited to this type of investigation since its large broad leaves can provide an ample supply of disks over the growing period. Moreover if one half of a very young leaf is removed longitudinally the other half will continue to grow normally, attaining in a week or two dimensions equal to, if not larger than, a normal half-leaf. In this way a leaf can be sampled shortly after emergence and the same leaf can be studied throughout a normal growing period.

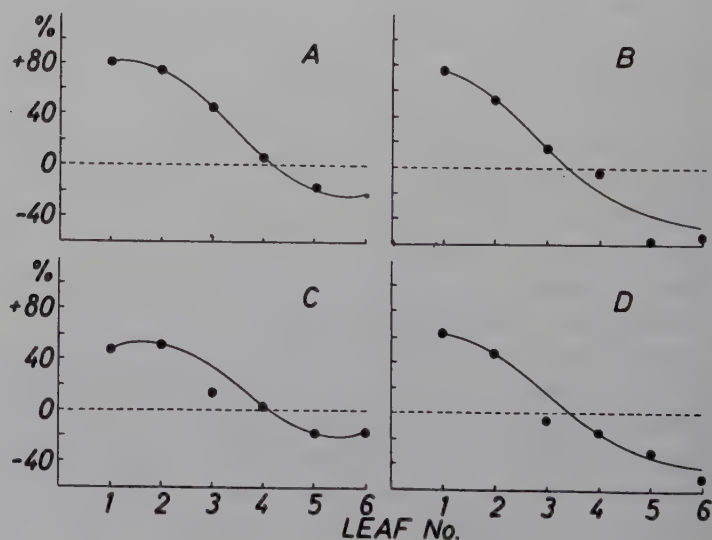


Figure 2. *Response of Successive Mustard Leaves to Various Inhibitors.* On the ordinate % inhibition (positive values and % stimulation (negative values).

Table 1. *Percentage inhibition of respiration recorded with leaves of varying age.*
The values denote QO_2 .

Leaf No. 1	Plant and inhibitor														
	<i>Zea mays</i> KCN 2×10^{-4} M			<i>Atropa belladonna</i> KCN 2×10^{-4} M			<i>Atropa belladonna</i> NaN ₃ 1×10^{-3} M			<i>Atropa belladonna</i> NaN ₃ 5×10^{-4} M			<i>Phaseolus multiflorus</i> NaN ₃ 5×10^{-4} M		
	Con- trol	KCN	% Inh.	Con- trol	KCN	% Inh.	Con- trol	NaN ₃	% Inh.	Con- trol	NaN ₃	% Inh.	Con- trol	NaN ₃	% Inh.
1	11.1	1.05	90.5	4.32	1.50	65.2	6.50	2.50	61.5	3.54	3.00	15.0	5.65	1.42	74.9
2	2.73	1.01	63.0	4.22	1.75	58.5	4.40	2.01	54.3	2.19	1.90	13.2	2.44	1.24	49.2
3	1.50	0.79	47.3	2.21	1.32	40.3	1.67	1.24	25.7	1.76	1.56	11.3	1.64	0.84	48.8
4	1.98	1.03	48.0	1.63	1.21	25.7	2.04	1.66	18.6	1.85	1.88	—	1.6	1.42	26.8
5	1.32	1.02	22.7	1.52	1.70	—	11.8	2.05	1.88	8.3	2.35	2.63	—	11.9	1.29
6	0.53	0.78	—	47.1	2.36	2.73	—	15.7	2.02	2.15	—	6.4	2.04	2.49	—

¹ Leaf numbers in this table do not indicate successive leaves of the plant which were chosen at random, but increase in number corresponds with increasing age. A minus sign denotes stimulation of respiration.

Two tobacco plants were sampled when they were bearing 12 visible leaves. The results of inhibition studies using 2×10^{-4} M KCN are shown in Table 3. Material from each leaf of the plant was used in both control and cyanide treated vessels so that an exact measure of the cyanide sensitivity of each leaf was obtained. The second plant, identical with the first, was used to replicate the results. The leaves were numbered from the apex downwards so that the higher numbers correspond with the first formed leaves. A longitudinal half was taken initially from the uppermost 3 leaves and disks were struck from the remainder. Subsequently disks were struck from the remaining halves of the first 3 leaves. After the first sample was taken at the end of June the plants were allowed to grow normally except that

Table 2. *The effect of inhibitors on young and old tomato leaf respiration.*

Inhibitor	Molar Concen- tration	QO_2					
		Old leaves			Young leaves		
		Control	+ Inhib.	% Inhib.	Control	+ Inhib.	% Inhib.
Cyanide	5×10^{-4}	2.12	1.23	42.0	3.13	0.63	80.0
Cyanide	25×10^{-3}	1.96	1.00	49.0	2.90	0.63	78.0
Dinitrophenol	1×10^{-4}	1.52	1.27	16.4	3.01	1.74	42.2
Dinitrophenol	1×10^{-3}	1.83	1.01	44.8	2.87	0.72	74.7
CO: O ₂ (80 : 20)	—	1.64	1.68	—	2.4	2.90	17.2
CO: O ₂ (80 : 20)	—	1.80	1.38	23.3	2.91	1.53	47.1
o-Phenanthroline	1×10^{-3}	1.58	1.50	5.1	3.00	2.29	23.7
Sodium Azide	1×10^{-3}	1.59	1.00	37.1	2.78	1.01	63.7

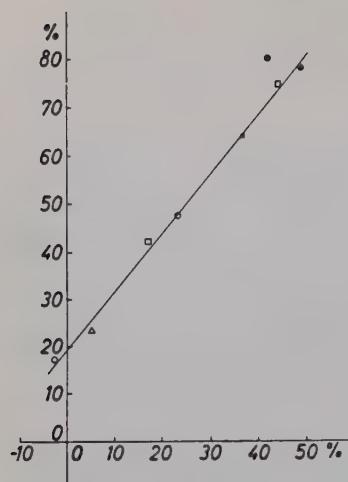


Figure 3. *Response of Old and Young Tomato Leaves to Various Inhibitors at Various Strengths Compared.* On the abscissa % inhibition of old leaves, on the ordinate % inhibition of young leaves. ● cyanide, □ dinitrophenol, ○ carbon monoxide, × sodium azide, △ o-phenanthroline.

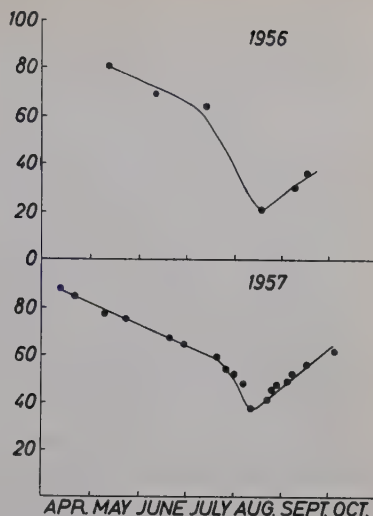
flowering shoots were removed. The last sample was taken at the beginning of August. In this way it was possible to follow the change in cyanide sensitivity vertically on the plant at any given time and horizontally (in respect of time) on any one leaf. It is apparent from Table 3 that the cyanide sensitivity of an individual leaf measured over a period of time and the cyanide sensitivity of a series of leaves taken from a plant at any one time follow the same trend. The changes in cyanide sensitivity of a young leaf as it ages is shown by leaves 1, 2 and 3.

3. *Effect of Potassium Cyanide on respiration of dwarf shoots of larch*

Table 3. *Effect of 2×10^{-4} M KCN on the respiration of successive leaves of tobacco at different times. % Inhibition.*

Date	Tobacco Plant A			Tobacco Plant B	
	25.6.56	12.7.56	3.8.56	27.6.56	1.8.56
Leaf No. 1	53.0	35.0	24.6	55.4	29.2
2	71.6	36.1	32.5	63.1	21.5
3	65.8	35.5	29.4	63.8	31.8
4	42.5	36.9	39.1	48.3	25.8
5	30.4	32.0	29.4	50.7	21.0
6	35.7	26.0	15.7	51.9	33.9
7	42.7	26.6	13.3	59.2	37.9
8	43.2	23.9	24.6	41.7	36.3
9	43.8	28.7	40.0	33.9	37.2
10	45.7	14.3	—	45.5	18.6
11	54.5	30.7	—	37.7	41.3
12	35.7	—	—	—	—
Average % Inhibition	47.1	29.6	27.6	50.1	30.4

Figure 4. *Percentage Inhibition by 1×10^{-4} M KCN of Oxygen Uptake of dwarf shoots of Larch during two growing Seasons.*



and cedar. Another technique whereby the senescent changes in leaf tissue may be investigated throughout a growing season without having to consider the complications of an ageing meristem or possible interference due to a changing position on the plant which might result in an altered nutritional supply, is to sample a plant where leaf emergence is contemporaneous over the entire shoot-system. This type of phyllotaxis is typical of most trees and larch provides a particularly convenient source of material. In the early spring larch produces a flush of dwarf shoots. Each dwarf shoot consists of 30—40 needles which continue to adhere together after the removal of extraneous bracts and, because of this, the shoot can be easily infiltrated and transferred to a Warburg vessel. Employing the sampling technique recommended for conifers by White (1954) representative sampling of dwarf shoots from 3 and 4 year old branches of 12—15 year old trees was commenced in the early spring when the young needles were just visible and continued until the late autumn when the needles were falling. The sensitivity of the shoots to 1×10^{-4} M KCN was followed over two growing seasons and the results are plotted in Figure 4. From an initial value of 80 % the percentage inhibition falls off gradually until the end of July when it reaches a very low level. During senescence the sensitivity redevelops.

In its production of dwarf shoots, the cedar tree resembles the larch but it differs in that the dwarf shoots are persistent, the needles generally lasting from 3—6 years. In any one dwarf shoot it is possible therefore to separate the needles, which are arranged in false whorls, into their various age groups and generally samples of needles aged from 1 to 4 years can be

Table 4. *Effect of 1×10^{-4} M KCN on the respiration of cedar needles of varying age.*

Age of needles	% Inhibition			
	<i>Cedrus atlantica</i>		<i>Cedrus libani</i>	
First year	90.1	87.6	82.3	82.5
Second year	44.4	30.0	9.7	12.1
Third year	7.7	5.2	6.7	10.3
Fourth and fifth year	6.2	— 2.3		

A minus sign denotes stimulation of respiration.

obtained. The cyanide sensitivity of needles from *Cedrus libani* and *C. atlantica* is recorded in Table 4.

4. *Influence of iron status on the response of leaf tissue to heavy metal inhibition.* To test the possibility that sensitivity to heavy metal inhibition might be determined by the iron status of the tissue some experiments were carried out with iron deficient and iron toxic leaves. The influence of iron status on the cyanide and carbon monoxide inhibition of the respiration of different leaves is shown in Table 5. Albino leaves of *Bougainvillea* have a high P/Fe and K/Ca ratio compared with normal leaves and consequently their nutritional status may be described as iron deficient (DeKock and Hall, 1956). These albino leaves are much more sensitive to cyanide and carbon monoxide than green leaves. On the other hand manganese deficient mustard leaves which are in fact iron toxic are less sensitive to heavy metal inhibitors than normal leaves and in this respect resemble old leaves such as senescent leaves of avocado (*Persea americana*) which accumulate iron and also are less sensitive to inhibition. The effect of 5×10^{-4} M KCN on normal, iron deficient and manganese deficient (*i.e.* iron toxic) plants of *Beta vulgaris* is also shown in Table 5.

Table 5. *Effect of inhibitors on the respiration of iron deficient and iron toxic leaves.*

Tissue	Inhibitor	Percentage inhibition
Bougainvillea (green)	25×10^{-4} M KCN	12.9
(albino)		23.4
(green)		48.0
(albino)		50.4
Mustard (normal)	CO: O ₂ (80:20)	— 3.0
(Mn deficient)		15.5
Avocado (young)		5.3
(old)		— 14.5
Spinach (normal)	5×10^{-4} M KCN	11.0
(Fe deficient)		— 8.9
(Mn deficient)		82.9
		87.7
		57.5

A minus sign denotes stimulation of respiration.

5. *Efficiency of vacuum infiltration.* Infiltration of the dicotyledonous leaves presented no difficulty and infiltration of the larch shoots was clearly visible. It was more difficult to decide whether or not the infiltration of the cedar shoots was complete because of their glaucous appearance. It was decided to test the efficiency of infiltration of larch and cedar shoots by comparison between the content of P^{32} in shoots which had been subjected to vacuum infiltration in a radioactive solution and those which had been merely shaken in the solution. The results clearly vindicated the efficiency of infiltration. In two different species of Larch, *Larix decidua* and *Larix leptolepis*, the ratio of P^{32} in infiltrated to shaken shoots was 30 : 1 and 31 : 1. Infiltration of cedar similarly confirmed that infiltration was effective with needles of all ages.

Discussion

1. *The sensitivity of successive leaves to inhibitors*

Kidd, West and Briggs (1921) were the first to show that the respiration rate of the plant as a whole decreased throughout its life, the rate at the termination of the life-cycle being approximately one tenth of the initial value. They showed too, that this downward pattern was the summation of decreasing respiratory activity of the stem, leaves and flowers, each type of organ manifesting a downward gradient which resulted, in part at least, from the fact that there was a continuous decline in the respiration of the meristematic tissue of the stem apex. Richards (1938) confirmed that each successive leaf formed at the stem apex was endowed with a respiratory activity lower than that of its immediate predecessor and the fact that each individual leaf commenced life with a physiological constitution which was not identical with that of any other leaf made him question the validity of experimental work which regarded a set of leaves present on a plant at any one time as constituting a true age series.

The technique of sampling a series of leaves from one plant was largely adopted in the work reported here because the investigation was concerned more with the differing response to inhibitors of leaves which could be broadly described as old in comparison with those which would, on the same diagnostic basis, be characterised as young, for which purpose it is unnecessary to assume that the leaves had originally the same constitution. Nevertheless, the fact that a similar trend is apparent when the cyanide sensitivity of the leaves of a plant such as tobacco is estimated by sampling all the leaves at one time or by sampling the same leaf at different times suggests that by adopting the former procedure the danger of obtaining grossly misleading results, at least in respect of the tissues' reaction to heavy metal

inhibitors, is not serious. Moreover the opinion that the cyanide sensitivity of a series of leaves, present on a plant at any one time, is indicative of the changes in sensitivity which occur in an ageing leaf is further confirmed by the experiments on dwarf shoots of larch which can be assumed legitimately to possess an identical physiological constitution and which as they age show a decreasing cyanide sensitivity similar to that obtained from a series of leaves present on a growing plant. Cedar shoots furnish additional corroborative evidence and although on a dwarf shoot of cedar the meristem is ageing from year to year the respiration of each season's new growth is inhibited 80—90 % with cyanide whereas after a year or two this sensitivity has been reduced to a very low value.

It may be concluded therefore that age profoundly affects the sensitivity of leaf tissue to heavy metal inhibitors and that although it has been opined that "it is impossible to predict what course any particular characteristic will take throughout the leaf series" (Richards, 1934) the sensitivity of a leaf to heavy metal inhibitors may be predicted from a knowledge of its position on the stem.

2. Possible causes of the lowered sensitivity to inhibitors of mature leaves

Several theories have been advanced to explain the unresponsiveness of mature leaf tissue to heavy metal inhibitors. Stenlid (1949) suggested that failure to inhibit oxygen uptake of old leaves with NaN_3 (Marsh and Goddard, 1939) was due to the unsuitability of the pH and to anatomical differentiation in old tissue resulting in an altered permeability. In the experiments reported here the pH was buffered at 6.0 and it is likely that some of the inhibitor was present in the form of the azide ion which is considerably less effective than the undissociated molecule. But even so inhibitions of up to 75 % were recorded in young tissue with $5 \times 10^{-4} M \text{NaN}_3$. Moreover the pattern of inhibition obtained from leaves of varying age with azide was similar to that obtained with cyanide and carbon monoxide, to neither of which do considerations of pH in the present circumstances apply.

In view of the precautions taken to ensure adequate penetration of the inhibitor into the tissue it is doubtful that permeability changes determine the tissue's response to the inhibitors. Infiltration of the dicotyledonous leaves presented no difficulty and the test of the efficiency of vacuum infiltration showed that old tissue was no less permeable to the experimental fluid than young tissue, even with cedar needles where there would be a certain amount of cuticularisation with age. In addition the molecular dimensions of carbon monoxide or potassium cyanide are not such as would hinder

the rapid penetration of these molecules into the leaf tissue. Indeed the fact that with increasing age there is an increasing stimulation of respiration is good evidence in support of the view that the molecules were present at the reactive sites and that their access to these sites was unimpeded by structural alterations associated with senescence.

Other explanations of the altered sensitivity to inhibitors of mature leaves suggested in one form or another by various workers (Marsh and Goddard, 1939; Albaum and Eichel, 1943; Webster, 1952; James, 1953; Daly and Brown, 1954) are: —

(i) Cytochrome oxidase although still functioning as the terminal oxidase is no longer the rate limiting step in respiration.

(ii) Cytochrome oxidase is wholly or partly replaced by some other non-metal oxidase.

The first of these can be regarded as a plausible theory to account for the ineffectiveness of cyanide inhibition of mature leaves but on the basis of this theory one would expect that a considerable increase in the level of inhibition would be obtained by using a greater concentration of the inhibitor. However as will be seen from Table 2 increasing the concentration of KCN from 5×10^{-4} M to 25×10^{-4} M only increased the level of inhibition in mature tomato leaves from 42 % to 49 %. Furthermore while the theory offers a possible explanation of the inactivity of the inhibitor it cannot account for its stimulatory effect on older tissue.

The second theory has been frequently invoked to explain the resistance of some tissues to heavy metal inhibitors although recently there has been some defection in the ranks of its advocates. The position has been weakened somewhat by the demonstration that many of the flavoproteins, considered formerly to be the most likely candidates for the role of cyanide resistant terminal oxidase, are metalloenzymes (Mahler, 1956); and Bonner (1957) has drawn attention to the fact that the oxygen affinity of flavoproteins is low in comparison to their affinity for hemoproteins and they would therefore operate preferentially and more efficiently in anaerobic electron transfer.

The pronounced insensitivity of certain tissues to cyanide inhibition suggests the occurrence of cyanide resistant respiratory pathways and several attempts have been made to isolate such a system. One of the most successful has been that of Martin and Morton (1955) who showed that microsomes isolated from silver beet petioles contain a cyanide insensitive pathway for the oxidation of DPNH and TPNH. A heme protein of the cytochrome b_3 type was associated with this pathway and as the rate of the reaction was too rapid to be the result of autoxidation they postulated the existence of a cyanide insensitive cytochrome b_3 oxidase. Bendall and Hill (1956) have demonstrated that a particulate fraction isolated from arum spadix, the

classical cyanide insensitive tissue, contains among other cytochrome components a large amount of one which they characterise as cytochrome b_7 and which they claim is an integral part of a cyanide resistant pathway. Yocum and Hackett (1957) presented further evidence for the participation of cytochrome in aroid respiration and they concluded quite independently that cytochromes of the b group were directly responsible for the cyanide stable respiration.

3. Possible causes of the stimulatory effect of inhibitors

The fact that there may be cyanide stable respiratory pathways does not, however, offer an adequate explanation of the stimulatory effect of respiratory inhibitors. Of the four inhibitors used in the present investigation 2,4-dinitrophenol is thought to increase oxygen uptake by uncoupling it from phosphorylation and although the inhibitory effect of azide is generally attributed to the chelating power of the liberated hydrazoic ion there is some evidence that it interferes with phosphorylation and so possibly might have an effect similar to that of dinitrophenol. Harley *et al.* (1956) have suggested that azide and cyanide may uncouple phosphorylation from respiration in excised beech mycorrhizas, and Griffiths and Hackett (1957) have suggested that carbon monoxide exerts an uncoupling effect on potato tuber slices. However, Calo and Varner (1957) have claimed that while the increased respiratory activity of aerated potato disks is linked to phosphorylation which can be uncoupled by dinitrophenol the phosphorylation is much less sensitive to cyanide.

One further possibility is that the stimulations caused by cyanide and carbon monoxide may be the result of the complexing and consequent removal of inhibitory heavy metals present in the cell. In a series of leaves such as that used in the present investigation well-defined changes in mineral content occur in the ageing leaves. DeKock (1955) has drawn attention to the significance of the P/Fe ratio in determining the metabolic state of the cell. Noack and Liebig (1940) studied the form in which iron occurred in plant cells and chloroplasts and showed that the bulk of it was present in the ferric form and was firmly bound to organic compounds. Only 8 % was present in the "active" form as ferrous ions. Holden (1952) has followed out the changes in phosphorus compounds of ageing tobacco leaves and has shown that between young small leaves and fully expanded leaves there is a decrease in the total P per gramme of dry matter and the greater part of the loss is in the form of organically bound phosphorus. Such a decrease would release iron bound in the ferric form and it would tend to accumulate in the form of free ferrous ions. For this reason the

respiratory activity of older leaves is not seriously affected by a decrease in available iron but the high respiration rate of the young leaf is very dependent on an adequate supply of iron and any deficiency of this element is immediately reflected in a decreased respiratory activity of young tissue (Glenister, 1944). Young tissue would therefore be expected to be very sensitive to heavy metal inhibitors such as cyanide and carbon monoxide but as the tissue became less dependent on iron availability (due both to a decreased respiratory rate and an increased iron supply) its sensitivity to chelating agents would decrease. As the leaves age the iron content increases steadily reaching a level as much as ten times that present in young leaves (Glenister, 1944). There is some evidence that this may amount to an iron toxicity which may exert an inhibitory effect on cellular respiration (Warburg, 1949). Heavy metal inhibitors by complexing or chelating this excess iron would therefore remove the inhibitory metal and so allow respiration to proceed at a higher level. If this were so the P/Fe ratio should give an indication as to the cyanide sensitivity of the tissue. The experiments with albino leaves of *Bougainvillea* which are iron deficient and manganese deficient leaves of mustard and spinach which are iron toxic furnish some evidence in support of this view (Table 5). A change in the iron status of the tissue might also have some effect on the character of the various cytochrome components.

Summary

1. Successive leaves of several plants have been shown to differ markedly in their response to cyanide, azide, carbon monoxide and 2,4-dinitrophenol. The respiration of young leaves is severely inhibited while that of old leaves may be markedly stimulated.

2. A comparison has been made between the cyanide sensitivity of individual tobacco leaves measured at different times during maturation and that of a series of leaves present on a mature plant. Both "ageing" series manifested an essentially similar response to cyanide.

3. Similar trends in cyanide sensitivity were obtained from ageing dwarf shoots of larch and cedar which are unaffected by alterations in nutritional supply resulting from a changing position relative to the stem apex.

4. Iron deficient leaves have been shown to be more sensitive to cyanide inhibition than normal leaves and iron toxic leaves to be less sensitive.

5. It is suggested that the iron status of the tissue may have an effect on its sensitivity to heavy metal inhibitors. The stimulation observed in old leaves in the presence of cyanide or carbon monoxide may be due to the complexing of some heavy metal which accumulates in ageing tissue and inhibits its respiratory activity.

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The Response of Various Species of Higher Plants to Light and Gibberellic Acid

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Gibberellic acid applied to etiolated Alaska pea seedlings reverses the light inhibition of stem elongation of these plants (Lockhart, 1956). This compound also reverses the red light inhibition of light-grown *Cosmos* (Lona, 1956). Another report has indicated, however, that application of gibberellic acid to light-grown beans promoted internode elongation independent of red or far-red light treatment (Downs *et al.*, 1957).

In an attempt to determine whether the gibberellic acid reversal of stem growth by radiant energy is a general phenomenon, several species of plants have been examined.

Methods and Materials

All plants were germinated in a darkroom maintained at $27 \pm 0.5^\circ\text{C}$. They were planted and grown in vermiculite in plastic pots and watered as needed with Hoagland's solution. As soon as a relatively large number of plants (8—10) appeared above the vermiculite in each pot, pots were selected, each containing several uniform plants. Two or more pots were selected at random for each experimental treatment. The selected plants were marked (if necessary) and any plants germinating after the beginning of the experiment were discarded.

Manipulations in the darkroom were performed under a 25-watt green incandescent light bulb placed 2 meters from the nearest plants. The first gibberellic acid treatment, as well as any subsequent treatments for the dark-grown plants was made in the darkroom with a supplementary, shaded 25-watt green bulb. Gibberellic acid was applied as a single 0.004 ml. drop of a 95 % ethanol solution of gibberellic acid

(Merck) to the growing point or young leaf of each plant. A gibberellic acid concentration of 1.0 mg./ml. was used, giving a dose of 4.0 μ g. per plant, unless otherwise specified. Indole-3-acetic acid (IAA) was applied in the same manner.

The light to which the plants were exposed was of two intensities. The higher intensity red light was obtained by radiating a bank of 4 48-inch pink fluorescent tubes (General Electric) through 2 thicknesses of 0.005-inch thick Du Pont red cellulose acetate. The tops of the plants were 10—20 cm. from the tubes. The frame holding the tubes as well as the upper sides of the ventilated cabinet were lined with aluminum foil. Blue light was obtained with 4 blue General Electric fluorescent tubes, screened through one thickness of 0.01 inch Du Pont dark blue cellulose acetate. A screen of gauze was fastened to the outside of the blue filter to reduce the intensity to that of the red source. The low intensity lights consisted of a single cool-white fluorescent tube filtered in the same manner as the high intensity lights above, and adjusted to equal intensities.

Measurements of light intensity were made with a Bowen photometer equipped with a no. 926 (type S-3) photoelectric cell. The sensitivity of this cell has been calibrated for the wavelength ranges used here. The light energy, measured with the aperture of the photocell normal to the source and at a distance equal to that of the plants, was $2,000 \text{ ergs} \cdot \text{cm}^{-2} \cdot \text{sec}^{-1}$ for the high intensity sources and $60 \text{ ergs} \cdot \text{cm}^{-2} \cdot \text{sec}^{-1}$ for the low intensity. The air temperature, measured with a mercury thermometer at the level of the plants, was maintained at $27 \pm 0.5^\circ \text{C}$ under the lights.

The two light sources were in the same room, and while every effort was made to prevent stray light from one source entering the other chambers it is probable that at least slight cross-contamination of light sources occurred. This had no effect on the responses to red light, since many experiments were carried out with red only. Stray red light appeared to have little effect on the plants grown in blue, since the red light treatments were not themselves saturating doses; and further, the formative effects, especially leaf development and node formation, were markedly delayed under the blue lights.

In the treatments reported here the plants either remained in complete darkness or were placed under the lights for the duration of the experiment, usually 2—4 days.

Experimental Results

Light and gibberellic acid. The effects of light and gibberellic acid on the stem growth of several plant species are illustrated in Figure 1. The selection of species to be examined in this way is, of course, necessarily restricted to plants having relatively large reserves of food. The results are typical of several experiments for each species.

It may be seen that *Helianthus annuus* (sunflower) is little inhibited by the light given here. Greenhouse experiments (where the temperatures were not comparable) suggested that the sunflower is further inhibited by full sunlight, but not to the same extent as other species. In the controlled temperature experiments, blue light had less effect on inhibition of stem elongation than red. This is in accord with the findings of many other workers (e.g.

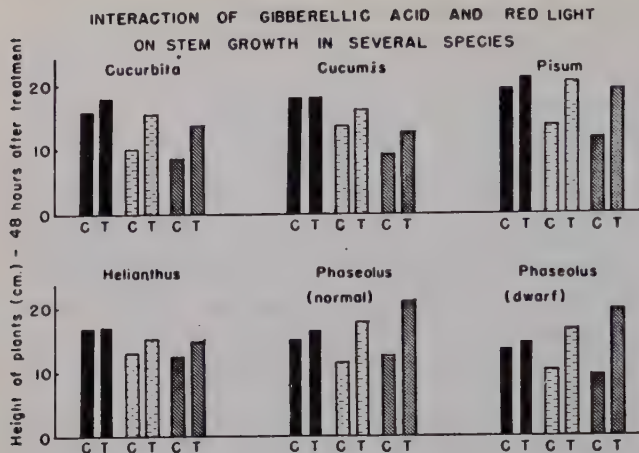


Figure 1. The effect of red light and gibberellic acid treatment ($4.0 \mu\text{g/plant}$) on stem growth of several species of higher plants. The light treatments consist of: black, complete darkness; light shading, low intensity red light; heavy shading, high intensity red light. "C" represents the controls and "T" the gibberellic acid treated plants.

Went, 1941; Downs, 1956) that red radiation is most effective for the inhibition of stem elongation of etiolated plants. The response of dark-grown *Helianthus* to gibberellic acid is negligible, while in light gibberellic acid restores growth nearly to the dark rate.

A comparison of two related species, *Cucumis sativus* (variety: National Pickling cucumber) and *Cucurbita pepo* (variety: Dark Green Zucchini squash) is of interest. The squash variety used here was found to be extremely sensitive to light. The inhibition of elongation approached 50 % of the control growth, and this inhibition could be achieved even with the low intensity ($60 \text{ ergs} \cdot \text{cm}^{-2} \cdot \text{sec}^{-1}$) red light. Low intensity blue light gave only partial inhibition. With the *Cucumis*, growth inhibition at least as great could be achieved, but only with the high intensity light. A partial reversal of the light inhibition by gibberellic acid could be demonstrated with both these plants, but complete reversal was not usually achieved.

The inhibition of stem growth of *Pisum sativum* (variety: Alaska peas) by low intensity red (orange) light has already been shown to be completely reversed by treatment with gibberellic acid. A preliminary report has indicated that gibberellic acid will also reverse the inhibition of stem growth caused by irradiation with green or blue light (Vlitos and Mendt, 1957). This report is confirmed in the present study, in so far as both low and "moderate" intensity red and blue light is concerned.

Stem growth of *Phaseolus vulgaris* is depressed by light, and gibberellic acid application in this case results in a rate of stem elongation greater than that in darkness. This has been found to be true of both dwarf and normal varieties. The response of *Phaseolus* to light and gibberellic acid has been studied in considerable detail and will be reported in a subsequent paper.

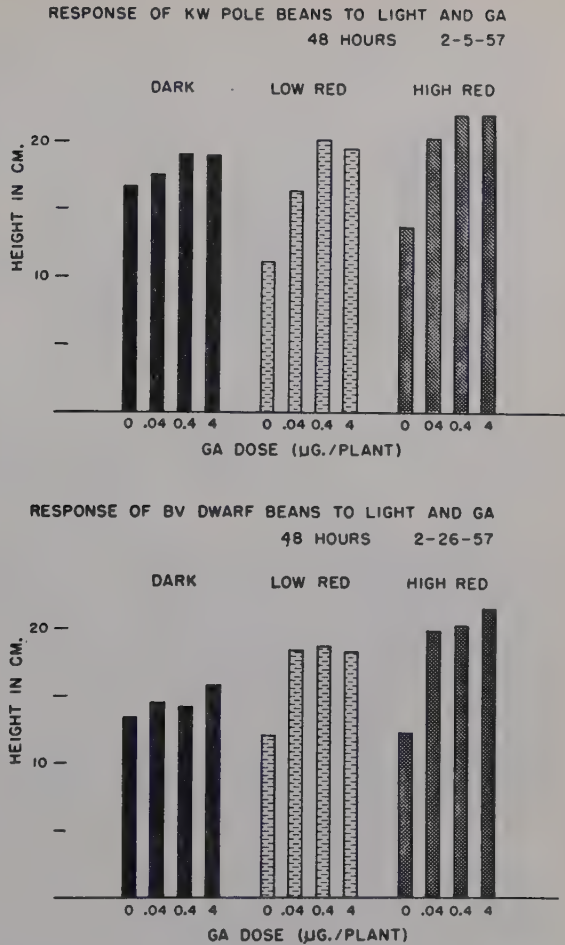


Figure 2. *Stem growth of Kentucky Wonder Pole and Black Valentine dwarf beans to various concentrations of gibberellic acid in darkness, low intensity, and high intensity red light. Measurement 48 hours after treatment.*

Typical results, demonstrating the effect of concentration of gibberellic acid is shown in Figure 2. In general, those plants which responded fully to the gibberellic acid treatments in light (*Phaseolus*, *Pisum*) gave a maximum response at a gibberellic acid treatment of 0.04–0.4 µg. per plant, while those plants which did not show complete interaction with light (*Cucurbita*, *Cucumis*) gave a maximum response only at higher doses of gibberellic acid (ca. 4.0 µg. per plant).

The response of all plants to blue radiation was examined and the response of *Cucurbita*, illustrated in Figure 3, is typical. In general, the response of the plants is similar to that in red light, but the blue is relatively less effective than red.

Light and indoleacetic acid. Considerable evidence has been reported in

RESPONSE OF CUCURBITA TO LIGHT AND GA

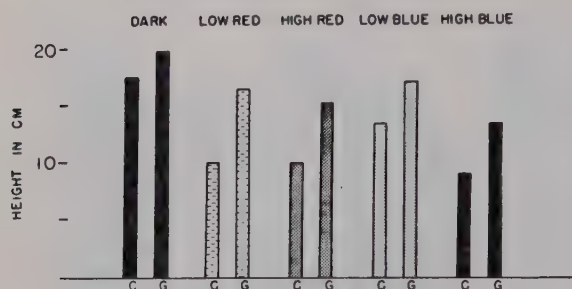


Figure 3. Stem growth of *Cucurbita pepo* in response to gibberellic acid (G) and various light treatments. Measurement 48 hours after treatment.

the past indicating an interaction between light and IAA (*e.g.*, Galston and Baker, 1953). It was considered possible that in cases reported here, where gibberellic acid failed to restore completely the dark-grown elongation rate to plants exposed to light, perhaps IAA in addition to the gibberellic acid might further increase the growth rate in light. Table 1 gives a typical result with *Cucumis sativus*. No promotion of growth by IAA application was found. It is interesting to note that doses of IAA which resulted in marked inhibition of growth in darkness resulted in little or no further growth reduction in the plants grown in the light.

Ultraviolet radiation, gibberellic acid and indoleacetic acid. Gibberellic acid has been found to be active in the reversal of the inhibition of growth of plants irradiated with visible light. Therefore it was considered of interest

Table 1. Effect of gibberellic acid and indoleacetic acid on the stem growth of *Cucumis sativus* in darkness and in red light. Measured 48 hours after treatment. Two separate experiments. Total height of plants in cm.

Indoleacetic acid treatment (mg./plant)	Dark		Light	
	—Ga	+Ga	—Ga	+Ga
0	16.1 ± 0.68 ¹	18.9 ± 0.49	9.6 ± 0.20	13.2 ± 0.56
0.004	18.2 ± 0.68	17.9 ± 0.35	9.4 ± 0.52	13.2 ± 0.33
0.04	17.8 ± 0.42	18.3 ± 0.49	9.1 ± 0.17	14.9 ± 0.44
0.4	16.6 ± 0.48	19.0 ± 0.42	9.5 ± 0.32	13.6 ± 0.37
4.0	16.9 ± 0.57	18.7 ± 0.56	9.8 ± 0.26	12.8 ± 0.39
0	17.3 ± 0.51	17.3 ± 0.64	9.0 ± 0.28	11.3 ± 0.49
4.0	15.0 ± 0.67	16.2 ± 0.54	10.3 ± 0.22	11.3 ± 0.57
40	13.5 ± 0.51	16.0 ± 0.69	9.5 ± 0.47	11.0 ± 0.59
400	11.8 ± 0.40	11.5 ± 0.44	9.5 ± 0.49	11.3 ± 0.54

$$1 = \sqrt{\frac{(\sum x)^2 - 1/n (\sum x^2)}{n(n-1)}}$$

Table 2. *The effect of gibberellic acid treatment on the ultraviolet inhibition of stem elongation.* Ultraviolet irradiation consisted of a 10-minute exposure to a 15-watt General Electric low pressure germicidal lamp at a distance of 10–15 cm.

Species	GA treatment ¹	Weight (in cm.), 48-hours after treatment		
		Dark control	Ultraviolet	UV screened ²
<i>Cucurbita pepo</i>	—	14.5 ± 0.96	9.4 ± 0.30	12.4 ± 0.48
	+	16.6 ± 0.50	10.5 ± 0.20	14.2 ± 0.81
<i>Phaseolus vulgaris</i>				
SG—dwarf	—	15.5 ± 0.57	9.6 ± 0.35	17.6 ± 1.22
	+	17.6 ± 0.73	10.2 ± 0.71	18.4 ± 1.13
<i>Phaseolus vulgaris</i>				
BV—dwarf	—	13.0 ± 0.37	8.2 ± 0.20	12.0 ± 0.46
	+	13.5 ± 0.57	8.6 ± 0.22	12.8 ± 0.57

¹ Gibberellic acid dose of 4 µg. per plant, as a 0.004 ml. ethanol drop.

² Screen consisted of a 3 mm. thick pane of window glass.

to determine whether gibberellic acid would also reverse the inhibition of stem elongation caused by ultraviolet irradiation. Ultraviolet irradiation has been shown to result in the inactivation of auxin, both *in vitro* and *in vivo* (Hare and Kersten, 1937; v. Denffer and Fischer, 1952; Brauner, 1953; Burkholder and Johnston, 1937), and it has been assumed that this could account for the inhibition of stem elongation by the ultraviolet radiation. The reversal of ultraviolet inhibited stem growth by IAA application has never been reported, although auxin applications have been shown to restore growth in plants which had received x-ray irradiation (Skoog, 1936).

The ultraviolet radiation was obtained from a 15-watt General Electric low pressure, germicidal mercury vapor lamp. This arc produces 80–90 % of its energy

Table 3. *Effect of gibberellic acid and indoleacetic acid on the stem growth of ultraviolet-irradiated Cucurbita pepo.* Measured 24-hours after treatment. Height of plants in cm.

Treatment	Dark	UV ¹	UV (screened) ²
Control	15.7 ± 0.44	8.1 ± 0.30	14.1 ± 0.28
Gibberellic acid ³	16.7 ± 0.54	9.3 ± 0.26	16.5 ± 0.50
Indoleacetic acid 0.004 mµg./plant	16.3 ± 0.42	9.0 ± 0.36	14.9 ± 0.36
0.04	15.4 ± 0.26	9.0 ± 0.14	15.6 ± 0.36
0.4	16.5 ± 0.26	8.7 ± 0.32	14.2 ± 0.10
4.0	16.3 ± 0.55	8.0 ± 0.20	14.5 ± 0.49
Gibberellic acid + IAA 0.04 mµg./plant	17.5 ± 0.36	9.1 ± 0.26	16.3 ± 0.33

¹ 10 minutes irradiation by a 15-watt General Electric germicidal, low-pressure mercury vapor lamp at a distance of ca. 10 cm.

² The same UV source screened through a 3 mm. pane of window glass.

³ dose: 4.0 µg./plant.

at the 254 m μ . line of mercury. No attempt was made to screen out the visible radiation; instead, a control was included in all experiments consisting of plants receiving identical radiation, except that it was screened through a 3 mm. thick pane of window glass. Such glass is known to transmit virtually no radiation below ca 320 m μ . The control growth in all cases confirmed the assumption that the inhibition was due to irradiation which could not penetrate the glass.

It was found that a 10-minute exposure to the unscreened ultraviolet radiation caused a marked inhibition of stem elongation, and this inhibition could not be overcome by the application of gibberellic acid (Table 2). A wide range of concentrations of IAA applied immediately following irradiation was also found ineffective in reversing the ultraviolet inhibition of stem elongation (Table 3).

Discussion

In those species whose growth in light is not completely restored by gibberellic acid, several possibilities exist to explain this lack of complete response. In all plants studied to date a marked interaction is observed between the light and gibberellic acid, even though complete restoration of growth is, in some cases, not achieved. This interaction indicates that even in those cases where growth is not completely restored to the dark-grown rate, nevertheless the natural gibberellin is involved in the light inhibition of growth. Results above indicate that application of IAA in addition to the gibberellic acid has no further growth promoting effect, at least in *Cucumis*.

Recently it has been shown that the gibberellins from higher plants differ from gibberellic acid and also differ between species (Phinney *et al.*, 1957). It appears possible that in some species the applied gibberellic acid may differ sufficiently from the natural hormone to account for its incomplete effectiveness. It may be noted that in just these species, larger doses of gibberellic acid are required for maximum response.

It was mentioned earlier that Downs *et al.* have published experiments with light-grown dwarf *Phaseolus*, in which they reported no interaction between the effect of light and gibberellic acid treatments, *on the growth of the second internode*. No statistical analysis was presented, and examination of the data suggests that an interaction may actually be present. It has been pointed out for peas (Lockhart, 1956) that the interaction of gibberellic acid and light is specifically on the total length of the stem, and not on the growth of individual internodes. Rather, light was found to have a specific effect on the rate of node formation, and gibberellic acid treatment had no effect on this process. Thus, in light gibberellic acid does not result in internode lengths equal to that in darkness (which would result in taller plants

in light than darkness), but rather increases the length of the individual internodes only enough to make the total height of the plant equal to that of darkgrown individuals. In the present paper only the total height of the stem, from the soil level to the terminal bud, is considered, although the usual light promotion of node formation was observed.

Summary

The stem growth of various species of plants which has been depressed by visible radiation is promoted by treatment with gibberellic acid. Growth in darkness shows little or no response to gibberellic acid treatment in these species. Where the restoration of growth in light is not complete, (e.g. *Cucumis*) IAA does not restore the balance of growth. It is suggested that reported differences in the natural gibberellins of higher plants may account for this lack of complete response.

Inhibition of stem growth by ultraviolet radiation was unaffected by either gibberellic acid or IAA.

Samples of gibberellic acid have been obtained through the courtesy of Merck and Company, Rahway, New Jersey; Eli Lilly and Company, Indianapolis, Indiana; and samples of gibberellin from the Kyowa Fermentation Industries, Tokyo. The author also wishes to acknowledge the technical assistance of Mrs. Renata Bracht. — The work was supported in part by the Herman Frasch Foundation.

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The Influence of Red and Far-red Radiation on the Response of *Phaseolus vulgaris* to Gibberellic Acid

By

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(Received October 22, 1957)

In the course of investigations on the interaction of light and gibberellic acid on the stem growth of various plants, it was found that light-grown dwarf *Phaseolus vulgaris* would respond markedly to gibberellic acid treatments. Dark-grown plants of the same varieties, however, showed little or no response to the same treatments. Since the response of *Phaseolus* was unique, in the author's experience, it was investigated further. The results of those investigations are reported here.

Methods and Materials

Seeds of various varieties of *Phaseolus vulgaris* (Ferry-Morse Seed Co., Los Angeles) were planted in plastic pots in vermiculite, and watered with Hoagland's solution. They were germinated in darkness at $27 \pm 0.5^\circ\text{C}$. Red light was obtained by irradiating a bank of 4 pink General Electric fluorescent tubes through 2 thicknesses of 0.005-inch red cellulose acetate, giving an intensity of $2,000 \text{ ergs} \cdot \text{cm}^{-2} \cdot \text{sec}^{-1}$ at the level of the plants. The far-red was obtained by irradiating 6 300-watt incandescent bulbs through 5 mm. Corning red-purple ultrafilters.

Experimental Results

Since light promoted the growth of the stem of *Phaseolus vulgaris* in response to gibberellic acid treatment, the minimum amount of light neces-

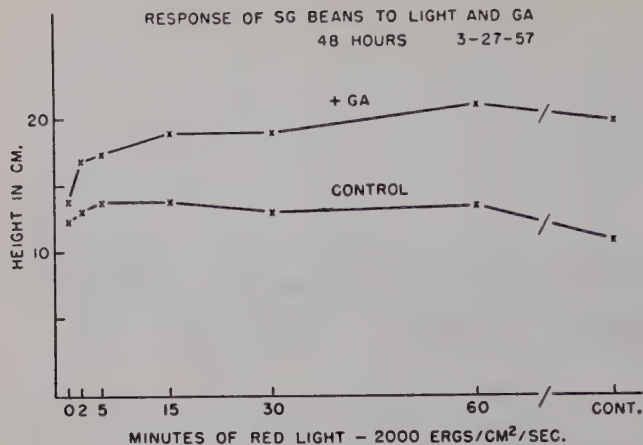


Figure 1. Response of dark-grown Stringless Green dwarf beans to a single treatment with red light and gibberellic acid ($4.0 \mu\text{g/plant}$). Measurement 48 hours after treatment.

sary to elicit this response was established. The results of a typical experiment are illustrated in Figure 1.

Seeds of the Stringless Green (SG) variety of dwarf *Phaseolus* were germinated in the darkroom at $27 \pm 0.5^\circ\text{C}$. When the seedlings were 6-days old they were treated with $4.0 \mu\text{g}$. gibberellic acid per plant, in darkness, and the plants which were to receive light-treatments were then removed to the light-room and exposed to the proper amount of red radiation, all at the same intensity. The plants were returned to the darkroom immediately after their light treatment was finished, and measured 48-hours later.

It may be seen that while the response of these plants to gibberellic acid in darkness is negligible, even a 2-minute exposure to the light results in a marked response to the treatment, and further increases in the duration of exposure to light gives further increases in the response of these plants to gibberellic acid. Further experiments demonstrated that a brief exposure to red light would result in a promotion of stem growth in all the dwarf and normal varieties of *Phaseolus* examined here.

Since brief exposures to red radiation resulted in a marked response to gibberellic acid, the effect of a subsequent irradiation with far-red was examined. The results of a typical experiment, using Black Valentine (BV) dwarf beans is shown in Figure 2.

The plants were grown in complete darkness. When they were 5-days old, selected plants were treated with gibberellic acid, as before. Treated and untreated plants were irradiated with 5-minutes red and/or 15-minutes of far-red radiation and immediately returned to the darkroom. The light treatments were repeated daily. The total height of the plants was measured 4-days later.

It may be seen that the growth of the gibberellic acid-treated plants which had been exposed to red light was markedly greater than that of the dark-



Figure 2. *Illustration of the response of Black Valentine dwarf beans to red or far-red radiation and gibberellic acid treatment. From left to right: plants grown in darkness, control and gibberellic acid treated; red light treated, control and gibberellic acid treated; and red followed by far-red radiation, control and treated.*

grown plants. When far-red irradiation immediately followed the red light treatment, the growth-promoting effect of the red light was completely negated. It has been found that the gibberellic acid is equally effective when applied either before or after the light treatment. It appears, then, that this response is a function of the same photomorphogenetic pigment that controls many other physiological processes (*cf.* Hendricks, 1956).

To determine something of the nature of the growth response to gibberellic acid, the effect of the length of time the red-far-red pigment remained in the far-red absorbing form was examined. Plants were exposed daily to 5 minutes of red light and then irradiated with far-red either immediately, after 6 hours, or after 24 hours (immediately prior to the next red light treatment). The results of a typical experiment are presented in Table 1. It may be seen that when the far-red is delayed for 24 hours it is without effect, but when the pigment remains in the far-red absorbing form for 6 hours an intermediate growth response is observed. Clearly the growth response is a function of the time the pigment is in the far-red absorbing form. The degree of variability in the growth of these plants makes it difficult to determine whether the response varies linearly with the time the pigment is in this form.

A surprising observation made in the present study was the finding that

Table 1. *The effect of the proportion of the time the red-far-red pigment is present in the far-red absorbing form on the growth response of Phaseolus vulgaris, variety Black Valentine to gibberellic acid treatment (4.0 µg/plant). Light treatments repeated daily. Height of plants (cm.)*

Treatment	Control	Gibberellic acid treated	Difference treated—control cm.
Dark	19.4 ± 0.62 ¹	24.4 ± 0.64	5.0
Red	23.8 ± 0.37	33.3 ± 0.44	9.5
Red + far-red (immediately) ...	23.9 ± 0.48	27.8 ± 0.62	3.9
Red + far-red (after 6 hours) ...	24.0 ± 0.31	29.9 ± 0.46	5.9
Red + far-red (after 24 hours)...	24.1 ± 0.29	32.5 ± 0.79	8.4

$$1 = \sqrt{\frac{(\Sigma x)^2 - 1/n(\Sigma(x^2))}{n(n-1)}}$$

dwarf varieties of *Phaseolus vulgaris* respond in a manner essentially identical to that of the normal (non-dwarf) varieties, when grown in darkness. Five commercial varieties of dwarf beans have been examined and all have been found to respond in a similar manner.

To determine whether these beans were actually normal and dwarf varieties, as they have been described, plants were observed growing in a normal greenhouse. The effect of gibberellic acid was also investigated. It was clear that the varieties were respectively dwarf and normal. The dwarf varieties responded to gibberellic acid as expected, the growth habit being changed from the bush to the pole habit of growth. It was of interest also to note a marked promotion of growth of the normal bean. This is consistent with the response of the normal bean to light and gibberellic acid, observed above, and adds further support to this observation.

Dwarf varieties of *Phaseolus* do not respond to gibberellic acid when grown in complete darkness, while it had previously been reported that a dwarf variety of *Pisum sativum* would respond markedly to gibberellic acid treatment when grown under the same dark conditions (Lockhart, 1956). In considering the possible explanations for this discrepancy it was considered possible that in the *Pisum* dwarf the red-far-red pigment, which acts in the control of stem growth, might be normally in the far-red-absorbing form, at least to the extent of not fully promoting stem elongation. Therefore, the effect of far-red radiation on the growth of dwarf *Pisum sativum* in darkness, and the effect of far-red radiation on the response to gibberellic acid treatment was investigated.

Seeds of *Pisum sativum*, variety: Morse's Progress no. 9, were germinated in complete darkness at 27°C. Six days after germination, plants were selected for uniformity and divided into 4 groups of ca. 20 plants each. Two groups were kept in

Table 2. *The effect of far-red irradiation and gibberellic acid treatment on the stem growth of dark-grown dwarf Pisum sativum, variety Morse's Progress No. 9. Plants measured 4-days after treatment. Height in cm.*

Treatment	Control cm.	Gibberellic acid treated
Dark-grown	15.0 \pm 0.27	23.5 \pm 0.12 ¹
Far-red irradiated	13.8 \pm 0.27	23.2 \pm 0.10

$$1 = \pm \sqrt{\frac{(\Sigma x)^2 - 1/n (\Sigma x^2)}{n(n-1)}}$$

darkness and the other two groups were irradiated with 15-minutes of far-red radiation (a dose of far-red radiation found fully effective with *Phaseolus*). One group from each treatment was treated with 4.0 μ g. per plant of gibberellic acid, and the other groups remained untreated. The experiment was repeated with identical results.

The results of this experiment are presented in Table 2. It is clear that irradiation with far-red has no effect on growth of these dwarf peas, or on their response to gibberellic acid. This experiment demonstrates, then, that the difference between the dwarf varieties of the two species is more fundamental than a difference in pigment steady-state condition, even though both dwarfs may be restored to the normal habit by gibberellic acid treatment when growing under natural conditions.

Discussion

The similarity of the response of normal and dwarf *Phaseolus* when grown in complete darkness, or with brief exposure to light, and when treated with gibberellic acid is striking. This suggests the possibility that dwarf varieties of *Phaseolus* show the dwarf characteristic only when grown in light. This in turn would imply that these dwarfs do not differ from the normals in their natural capacity to produce gibberellin; rather, it would appear that in high-intensity light the gibberellin is destroyed more rapidly, is less effective, or that production is decreased in light more markedly in the dwarf.

Such an hypothesis could only be fully verified by comparing the growth rates, in darkness, of dwarf and normal plants which differed genetically only by the single dwarf gene, much as Phinney (1956) has done for dwarfism in *Zea mays*.

A further conclusion, as a result of the experiments reported here, appears to be justified. Maximum stem growth of *Phaseolus* can take place only as

a result of treatment by red light. This response is not ordinarily observed, however, because the red light also serves to make the natural gibberellin in the plant the limiting factor for growth. It is only when the gibberellin system is saturated by an external source of gibberellic acid that the promotive effect of the radiation becomes evident. This response to red light is apparently not a general one, as evidenced by the absence of this response in all of the other species thus far examined. Presumably some factor is limiting growth in other species other than either the gibberellin or the red light factor. In *Pisum sativum*, variety Alaska, this is quite clearly the caulocaline factor (or group of factors) demonstrated by Went (1938), previously shown to be distinct from gibberellin (Lockhart, 1957).

Summary

Both dwarf and normal varieties of *Phaseolus vulgaris* fail to respond to gibberellic acid when grown in complete darkness. Brief exposure to red light causes both types of plants to elongate markedly in response to gibberellic acid applications. The effect of red light is reversed by subsequent exposure of the plants to far-red radiation. Stem elongation in response to gibberellic acid treatment in light is substantially greater than growth in darkness, indicating that the growth of these plants is *promoted* by light when gibberellin is made non-limiting.

Samples of gibberellic acid have been obtained through the courtesy of Merck and Company, Rahway, New Jersey; Eli Lilly and Company, Indianapolis, Indiana; and samples of gibberellin from the Kyowa Fermentation Industries, Tokyo. The author also wishes to acknowledge the technical assistance of Mrs. Renata Bracht. This work was supported in part by the Herman Frasch Foundation.

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Use of Chelation Phenomenon in Studies of the Structure and Action Mechanism of *Oscillatoria* Phosphorylase

By

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(Received October 25, 1957)

Introduction

It is becoming increasingly evident that an important mode of action of enzymes in general may well be through their union with metallic ions and the subsequent effect of the *chelate* thus formed in altering the substrate (Martell and Calvin, 1952; Westheimer, 1955; Granick, 1957). That enzymes may operate mainly through the alteration of chemical bonds of a substrate via ligation with an *essential* metal is well known for certain proteolytic enzymes which depend on a temporary union with magnesium ions (Martell and Calvin, 1952) and for the various oxidative enzymes which depend on a permanent union with iron or copper ions (Warburg, 1948; Dawson, 1950).

The effect of chelation on the polyglucoside-synthesizing enzymes, *phosphorylase* and *branching enzyme*, of the blue-green alga, *Oscillatoria princeps*, was reported in a previous study (Fredrick, 1957). The mechanism was postulated to be one of chelation of probable toxic metallic ions such as copper, thereby causing what appeared to be an *activation* of the enzymes, and subsequently, with increasing concentrations of chelating agent, an *inhibition* of the enzymes probably as a result of the sequestering of essential metals (in this case, calcium, manganese, and possibly iron).

The factors which would necessarily be involved in this inhibition would be those directly concerned with the relative strength of the chelation bonds

between the enzyme and essential metal and the essential metal and the added chelating agents. In order to verify this hypothesis, it was decided to use chelating agents derived from one of the chelating materials used in the previous study, kojic acid, or 2-hydroxymethyl-5-hydroxy- γ -pyrone.

It is known that a measure of the stability of a given chelate is related to the oxidation potential of the chelate (Calvin and Bailes, 1946), and that the effect of substituents in the ligand or chelating agent influence the relative strength of the bonds between metal and ligand. Hence, it was decided to test the inhibitory effect observed with kojic acid in the previous study (Fredrick, 1957) with *Oscillatoria phosphorylase* as the enzyme.

Experimental

Various derivatives of kojic acid were chromatographed on Whatman no. 1 filter paper by ascending method in a developing solvent mixture made up of 70 parts of methyl ethyl ketone, 20 parts of *N*-methyl-2-pyrrolidone, and 10 parts of water containing 0.1 per cent ammonium acetate. After 2 hours at 25° Centigrade, the paper was air-dried and sprayed with 0.2 per cent aqueous ferric chloride solution. The chromatogram was dried in an oven for 3 minutes at 80° Centigrade. The spots were easily visible and the R_f values were constant over a suitable range.

Using this method, each derivative was tested for purity to insure non-contamination with other kojic acid compounds. It was found that only two of the derivatives required further purification by recrystallization from hot ethanol. All the derivatives used were ascertained to be chromatographically pure. The derivatives were used in those concentrations which gave a definitive inhibition for kojic acid, 0.001 Molar (Fredrick, 1957).

Phosphorylase was prepared from extracts of normal cultures of *Oscillatoria princeps* as described (Fredrick and Mulligan, 1955). The derivatives were added directly to equal quantities of enzyme solution, $\frac{1}{2}$ hour prior to addition of reaction mixtures to the enzyme solutions. The reaction mixtures consisted of buffered substrate (glucose-1-phosphate) as described (Fredrick, 1951). The amount of inorganic phosphate split from the substrate, as well as the glucose value after isolation and hydrolysis of the polysaccharide formed, were used to follow the course of the reaction (Fredrick, 1951, 1952).

The iron chelates were formed by using a three to one ratio of ligand to ferric salt (Pfizer & Co. 1956). Oxidation potentials were determined by means of a platinum electrode against a standard calomel electrode with a Beckman model G pH meter. Corrections were applied to these observed e.m.f.'s to convert them to normal hydrogen electrode potentials.

Those reactions where derivatives were added to the enzyme were run parallel to uninhibited enzyme reactions where distilled water was added to the enzyme. Incubations were at 29° Centigrade at a pH of 6.95. Comparisons of the inhibited reactions with the uninhibited reactions were made after 60 minutes incubation. This had previously been established as the equilibrium point (Fredrick 1954) for this reaction.

Figure 1. Structures of Kojic Acid Derivatives.

Derivative and Abbreviation	Structure	Mol. Wt.
2-hydroxymethyl-5-hydroxy- γ -pyrone. (K.A.)		142
2,6-hydroxymethyl-5-hydroxy- γ -pyrone. (6 K.A.)		172
2-acetoxymethyl-5-hydroxy- γ -pyrone. (A.K.A.)		204
2-methyl-5-hydroxy- γ -pyrone. (A.M.)		126
2-chloromethyl-5-hydroxy- γ -pyrone. (C.K.A.)		160
2-bromomethyl-5-hydroxy- γ -pyrone. (B.K.A.)		205
2-hydroxymethyl-5-hydroxy- γ -pyridone. (K.A.P.)		141
2-octadecyl dimethyl methyl-5-hydroxy- γ -pyronyl ammonium chloride. (18-D.)		455
6,6'-dikoijyl methane. (D.K.)		296

* Approximate structure, not known definitely.

* *Physiol. Plant.*, 11, 1958

Table 1. *Paper chromatography of kojic acid derivatives.*

Derivative	Color	R _f	Sensitivity (μg)
A.M.	Violet-red	0.98	3
C.K.A.	Bright-red	0.92	4
B.K.A.	Dark-red	0.87	4
K.A.	Brown-red	0.62	3
A.K.A.	Violet-red	0.37	3
6 K.A.	Violet-red	0.34	3
18-D.	Orange-brown	0.22	6
K.A.P.	Violet	0.10	4
D.K.	Amber-brown	0.05	8

Results

The results of the paper chromatography studies are shown in Table 1. Note that the differences in R_f values allow easy separation of the various derivatives. The color of the spot, actually the color of the ferric-kojic acid derivative chelate, is fairly specific for each compound. The structure of these compounds is shown in Figure 1.

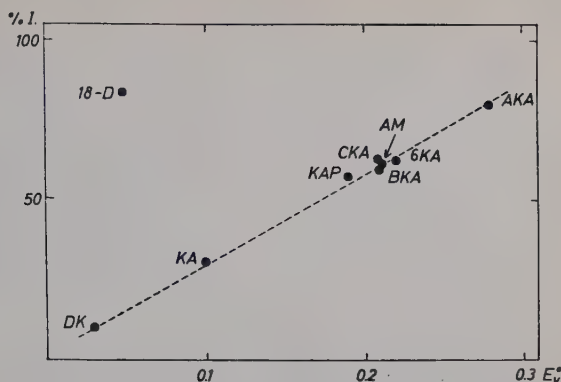
The oxidation potentials are shown in Table 2. The value for the hydrated ferric ion is given for comparison. Note that chelation has taken place with each derivative by the increase in electrode potential as compared to the hydrated ferric ion (Martell and Calvin, 1952).

Figure 2 shows the per cent inhibition observed with the various derivatives. As can be seen, five of the derivatives show an inhibition of from 55—65 % of phosphorylase activity. The relationship between the per cent inhibition and electrode potential appears to be a linear one, except for the quaternary derivative, 18-D. The reasons for this anomaly will be covered in the following section; they are mainly concerned with micelle formation (Fredrick, 1957).

Table 2. *Oxidation potential of kojic acid derivatives (iron chelates).*

Derivative	E° (volts)
A.K.A.	0.281
6 K.A.	0.223
A.M.	0.212
C.K.A.	0.211
B.K.A.	0.211
K.A.P.	0.195
K.A.	0.100
18-D.	0.051
D.K.	0.032
Hydrated Fe ³	— 0.771

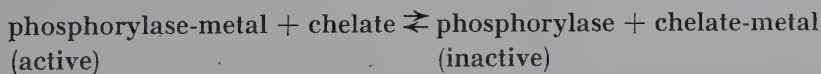
Figure 2. Inhibition of *Oscillatoria* phosphorylase as a function of chelate stability. Ordinate shows the % observed inhibition, and abscissa shows the electrode potential of the various kojic acid derivatives (Fe^3 chelates) corrected to standard hydrogen electrode values.



Discussion

An examination of the electrode potentials of the Fe^3 chelates of the kojic acid derivatives used in this study shows that these derivatives may be classified on the basis of chelate stability (Table 2). When these values are plotted against per cent inhibition, a linear relationship is apparent (Figure 2). The higher the electrode potential, and hence the more stable the chelate (Martell and Calvin, 1952), the greater the inhibition of *Oscillatoria* phosphorylase. It becomes possible to extract from this relationship some basic concepts with regard to phosphorylation.

Assuming that the situation with regard to *Oscillatoria* phosphorylase is such that a *phosphorylase-metal* chelate is necessary to enable the enzyme to form α -1 : 4 glucosidic linkages between dextrin and glucose-1-phosphate, then, if a chelating agent is added to the enzyme whose relative chelate-forming ability with the metal is greater than that of the enzyme's, it is apparent that a withdrawal of metal from the *enzyme-metal* chelate will occur, and that this would undoubtedly affect the catalytic properties of the enzyme (inhibition). Because of the unknown groups participating in the *phosphorylase-metal* chelate bond, the exact structural configuration of this enzyme chelate is obviously impossible to delineate at this time. However, it should be possible, by a study of the exactly balanced system:



to determine certain fundamental properties of these bonds. Of course, such a task would be a formidable one; many factors enter into such a study, not the least of which is the effect on chelate stability of substituent groups in the ligand (Martell and Calvin 1952).

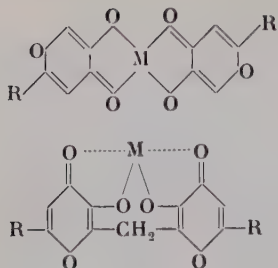


Figure 3. Probable structures of divalent metal chelates of kojic acid derivatives. The top figure shows the structure of kojic acid chelate. Note that it contains 2 molecules of ligand per divalent ion and has the formula $M(KA)_2$. The bottom figure shows the probable structure of the D.K. chelate, with the formula $M(DK)_2$, since confirmed by Job analysis (see Figure 4). The bonds in the D.K. chelate are much longer and much more strained than those of the stable K.A. chelate. ($R = -CH_2OH$)

It has been reported by Calvin and Bailes (1946), that these groups may influence the stability or "power of chelation" in a variety of ways. For example, in their studies using the substituted anils of salicylaldehyde, they found that electron-withdrawing groups such as $-NO_2$ or $-SO_3Na$ weaken the stability of these salicylaldehydes, while electron-donor groups such as $-OCH_3$ and $-CH_3$ strengthen the metal-chelate bond. In general, substituents in the ligand may effect the stability of the chelate by either influencing the acidity of the donor groups (those groups directly involved in the metal-ligand bond) and thereby influence the *resonance* of the chelate ring, or the substituent groups may cause steric effects that prevent the ligand molecules from assuming their most favorable orientation around the central metal ion (Martell and Calvin, 1952).

These effects are apparent in the data obtained in the present study. Note that the inhibitory power of each kojic acid derivative is directly related to the stability of the chelate formed (cf. Figure 2). The one anomalous compound was 18-D, which is a quaternary derivative. As was shown in a previous study (Fredrick, 1957), micelle formation by this particular compound directly affects its behavior, and hence, it will not be considered in the following discussions.

However, it is obvious from Figure 2, that such groups as $-CH_2OCOCH_3$ (an ester linkage), $-CH_3$, $-Cl$, and $-Br$, make for a more stable chelate (insofar as oxidation potentials are concerned), and thereby have the greater inhibitory effect on phosphorylase. Particularly interesting, in this respect, is the comparison of the inhibitory powers of the 2-methyl (A.M.) and 2-hydroxymethyl (K.A.) derivatives with regard to their respective structures (see Figure 1). The A.M. derivative has an electrode potential about twice as positive as that of the K.A. derivative (see Table 2). The inhibition of Oscillatoria phosphorylase by K.A. is about 31 per cent, while that of A.M. is 61 per cent, or approximately twice as great.

Another interesting observation along these lines is the effect on the metal ligation caused by substitution of the ether oxygen atom of the γ -pyrone

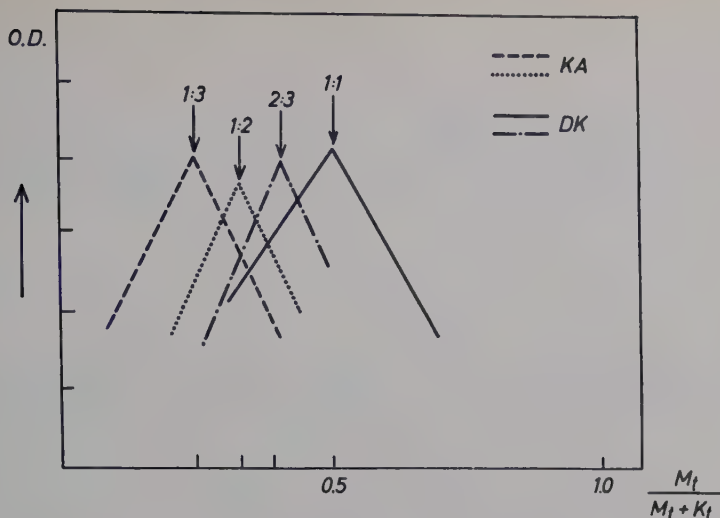


Figure 4. Job analysis of the Fe^{2+} and Fe^{3+} chelates of K.A. and D.K. Note that the ratio of metal to ligand in the case of the divalent Fe^{2+} is 1:2 for K.A. (....), and 1:1 for D.K. (—). With the trivalent Fe^{3+} , the ratio is 1:3 for K.A. (- - -), and 2:3 (or 1:1½) for D.K. (—). (Fredrick — *In preparation*).

nucleus by nitrogen, forming the analogous pyridone (see Figure 1). The effect of the nitrogen is to give the chelate a greater stability (see Table 2), and as a result, the γ -pyridone is a much more effective inhibitor than the γ -pyrone structure (see Figure 2).

When, on the other hand, a hydroxymethyl group ($-\text{CH}_2\text{OH}$) is introduced into the kojic acid molecule directly opposite the 2-hydroxymethyl group already on the molecule, such as in the 6 K.A. derivative, there is achieved a balance of the two groups (equivalent to a cancelling-out effect) and the derivative gives an inhibition similar to the 2-methyl derivative, A.M. The effect on the stability (see Table 2) is of the same order.

The behavior of the D.K. derivative (see Figure 1 for the probable structure) is obviously due to a straining of the D.K.-metal bonds because of *steric hindrance*. As a result, this derivative has a low electrode potential and a corresponding low order of phosphorylase inhibition (see Figure 2). The steric effect caused by this derivative is apparent if one takes into consideration the structure of the chelate-metal complex. For example, the structure of the kojic acid-metal chelate with a divalent metal is probably of the planar type (O'Kane, 1949; Wiley *et al.*, 1942) with the divalent metal bonds in ionic structure (see Figure 3). Now, with the D.K. derivative, the bonds formed with a divalent metal would be strained (see Figure 3) and hence

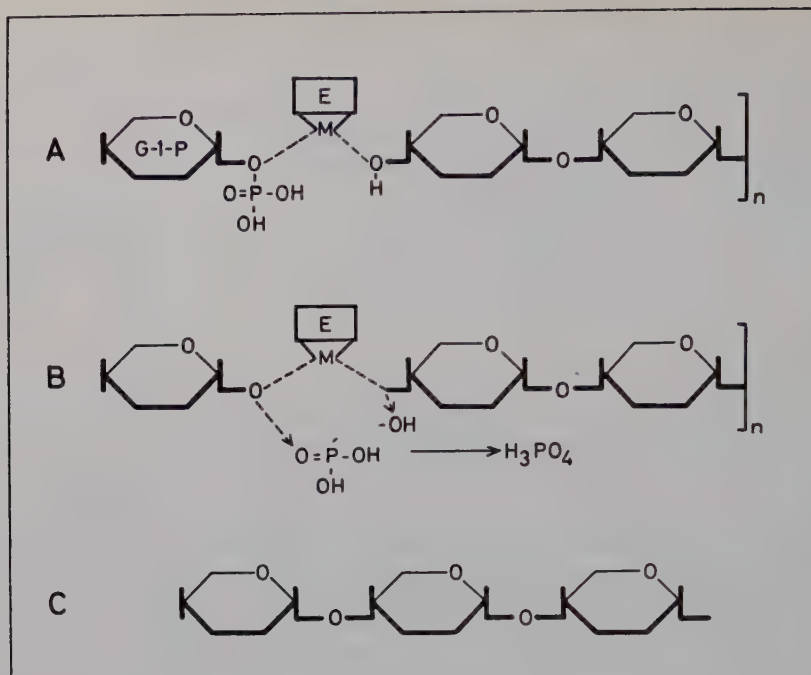


Figure 5. *The mode of action of Oscillatoria phosphorylase.* A shows the establishment of a 3-unit chelate between the phosphorylase (E), Metal (M), glucose-1-phosphate (G-1-P) and the linear maltodextrin primer where "n" is at least five residues in length (see Fredrick and Mulligan 1955). B, shows the release of the H_2PO_3 radical by the glucose-1-phosphate as a result of the chelate-binding, and also the simultaneous release of the hydroxyl group of the 4-carbon of the end residue of the "primer" dextrin. As can be seen, the recombination of these two radicals yields phosphoric acid. C, shows the resulting maltodextrin with the new alpha 1:4-glucosidic bond established. The new dextrin now contains "n+1" glucose residues, etc.

make for instability of the structure. With a trivalent metal such as Fe^3 or Mn^3 , the bonds formed by the 6,6'-dikoijyl methane derivative would be subject to even more strain, and hence, could be easily disrupted. This is apparent from the electrode potential of its Fe^3 chelate as shown in Table 2.

The inhibition of phosphorylase by D.K. is very slight. In fact, it begins to approach those levels which should yield valuable information as to the actual *enzyme-metal* chelate bonding.

Although no attempt was made in this preliminary study to refine the oxidation potential data, it may be used in a qualitative manner with regard to the following extrapolations as to the strength or stability of the *phos-*

phorylase-metal bond. It would appear, for example, that the metal(s) chelated by phosphorylase, though apparently stable to dialysis (Fredrick, 1951, 1952, 1955), nonetheless can be *exchanged* by the kojic acid derivatives used in this study, even by the D.K. derivative. Therefore, it would appear that the oxidation potential involved in the *phosphorylase-metal* bond must be of the order of that of the D.K. derivative. In other words, the reaction would be:



and the shift in equilibrium, of such dimensions as to give a detectable inhibition (approximately 10 % of total activity of uninhibited phosphorylase-metal). This is by far, the lowest degree of inhibition caused by any of these derivatives, but, nevertheless, it is indicative that an *exchange* of metal has taken place between the phosphorylase and the D.K. derivative.

Although this is not the first time that biological assay methods have been applied to chelates (Hastings *et al.* 1934), it is felt that this method offers a means whereby the ultimate structure of enzymes, and a better understanding of the so-called "catalytic action" mechanism can be attained.

Conclusions

1. The inhibition of phosphorylase by various derivatives of the chelating agent, kojic acid is described with regard to the stability factors effecting the chelate.
2. A linear relationship is shown to exist between the degree of inhibition and the stability of each chelate.
3. The possibility is explored of ascertaining certain facts as to enzyme-metal structures with regard to *Oscillatoria* phosphorylase. Such a method would be dependent on reaching the exact oxidation potential whereby the metal ion bound by the enzyme can be easily and reversibly exchanged with an added chelating compound.

I wish to express my thanks to the Chas. Pfizer & Co. organization, and particularly to William Bell and Harold B. Conant of its Brooklyn, N.Y. laboratories for making available most of the derivatives of kojic acid used in this study.

I also wish to thank the Antara Division of General Aniline and Film Corporation, New York City, for their gift of *N*-methyl-2-pyrrolidone used in the chromatographic portion of this study.

Last, but not least, I wish to thank Professor Arthur E. Martell, Department of Chemistry, Clark University, Worcester, Massachusetts, for his patient and informative letters.

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Gross Respiratory and Water Uptake Patterns in Germinating Sugar Pine Seed

By

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Introduction

Studies of germinating seeds have generally dealt with limited aspects of the germination phenomena and have frequently failed to relate interdependent changes. The purpose of this study was to analyze certain related changes occurring during the initial germination stages of sugar pine seed (*Pinus lambertiana*, Douglas). Measurements were made of the water uptake, dry weight changes, and the concomitant gas exchange pattern of the embryo and endosperm (female gametophyte) of stratified and non-stratified seeds during germination.

Sugar pine seeds require a prior treatment such as stratification for maximum germination (Jacobs, 1925). In this study the observations were limited to that period when the embryo is dependent upon the associated endosperm for its nutrient substrates. Germination is considered as beginning when the physiological changes induced through increased respiration result in growth of the embryo (Toole, *et al.* 1956).

Material and Methods

Materials. The experiments were carried out on seeds harvested in 1953 at Pino Grande near Placerville, California. All seeds were derived from cones of one tree. The cones were dried at about 70°C until the cone scales had reflexed and the seeds

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² Maintained at Berkeley, California, by the Forest Service, U. S. Department of Agriculture in cooperation with the University of California.

dropped, or could be shaken out. Seeds were then dewinged, air-dried to a moisture content of approximately 10 percent, and stored over CaCl_2 in a desiccator at 5°C until used.

Methods. Seeds were prepared for germination by soaking in a bromine-water solution for 5 minutes, and rinsing six times in sterile distilled water. The hard seed coat and inner papery membrane were then aseptically removed (Stone, 1948). For germination studies of excised embryos, the embryos were then removed aseptically and transferred to moistened filter paper in petri dishes, which were placed in an incubator at $30^\circ \pm 1^\circ\text{C}$. When the embryos were allowed to germinate in the endosperm, the divested seeds were planted $1/2$ inch deep in sterile vermiculite in pint wax containers with drain holes punctured in the bottom. The containers were watered to saturation and placed in a 30°C incubator. Seed stratification before germination was done by placing seeds in moist vermiculite at 5°C with the seed coats intact for intervals up to 3 months. Upon removal, before germination, the stratified seeds were treated in bromine solution as above.

For gas exchange measurements, the individually germinated embryos, after a specific period of germination, were placed directly in respirometer flasks. The embryo and endosperm of seeds germinated in vermiculite were separated before being placed in respirometer flasks. The bottom of the respirometer flasks contained sterile moist filter paper. To ensure comparable results, it was necessary to select embryos and seeds which had germinated to approximately the same stage of development, as indicated by radicle length.

Oxygen uptake was measured in standard Warburg flasks of approximately 6 ml. volume, with .04 ml. of 5 M KOH in the center well (Umbreit, *et al*, 1949). Embryo or endosperm samples were placed in the flask on moist filter paper. The flasks were then attached to a manometer and placed in a circular Aminco-Lardy Warburg apparatus at 30°C . After at least 10 minutes equilibration, the initial reading was taken. The manometers were shaken 112 oscillations per minute. Pressure changes were recorded at either 10 or 15 minute intervals for at last one-half hour.

Carbon dioxide evolution was determined by two methods. The first was the Warburg direct method which requires a comparison of two different samples (Warburg, 1914). To circumvent the many difficulties inherent in this method (Dixon, 1951), a modified respirometer flask was employed (Stanley and Tracewell, 1955). This second method permitted approximately simultaneous measurement of oxygen and carbon dioxide exchange in the same seed samples, a value previously unattained in studies involving germinating seeds.

'Bound' CO_2 measurements were made by placing duplicate tissue samples of known initial wet weight and final dry weight into Warburg flasks of approximately 12 ml. volume. The flasks contained 2.5 ml. of 1 N H_2SO_4 in the main compartment. The embryos or endosperm halves were balanced on a moistened filter paper disc which covered the center well. After 30 minutes had been allowed to reach equilibrium, the manometer was removed from the bath and tilted so that the seed component fell into the acid solution. An additional 40 minutes was allowed for the acid to penetrate throughout the tissue. The positive increase in pressure, multiplied by the flask constant, represents the number of μl of bound CO_2 released.

Moisture determinations were made with a Roller-Smith torsion balance. Surface water was removed from samples exposed freely to liquid before weighing by gentle pressing between two layers of absorbent tissue (Brown, R., 1943 a). Samples were dried to a constant weight at 80°C .

Results

Water uptake. The amount of moisture which must be present for a seed to germinate varies from species to species (Crocker and Barton, 1953; Walter, 1955). The rate of germination of sugar pine seed at 30°C, without the testa (the hard outer seed coat) and inner membrane, was influenced by the amount of moisture initially available (Figure 1). In these experiments the higher concentration of agar limited the water available to the seed (Brown, R., 1943 b). Thus, seeds germinated sooner on 0.5 percent agar than on 1.0 percent agar.

Stiles (1948), working with corn and cotton seeds, showed that components of those seeds hold water with a different tenacity. Figure 2 relates the percentage of water absorbed by different organs of the seeds of sugar pine. Although the embryo is the smallest of the seed components studied here, it absorbed the greatest percent of water on a dry weight basis. The percent water absorbed appears constant after 30 days exposure to a moist condition; however, moisture within the seed is redistributed after 60 days stratification.

The percentage of moisture absorbed by the embryo germinated in the endosperm alone increased with length of exposure to moist conditions (Figure 3). However, in unstratified seeds a plateau was reached soon after exposure to moist conditions. Only after an interval of approximately 40 to 50 hours at this water level, did a further increase in water absorption occur. The plateau was briefer and later in appearance in seeds stratified 60 days; and absent in the pattern of water absorption in seeds stratified 90 days.

Various kinds of non-living seed tissue were compared as to their capacity

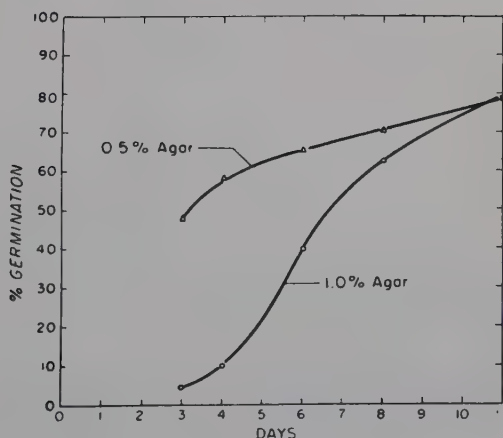


Figure 1. Effect of available moisture on rate of germination of *P. lambertiana* seed without testa and inner ptery membrane.

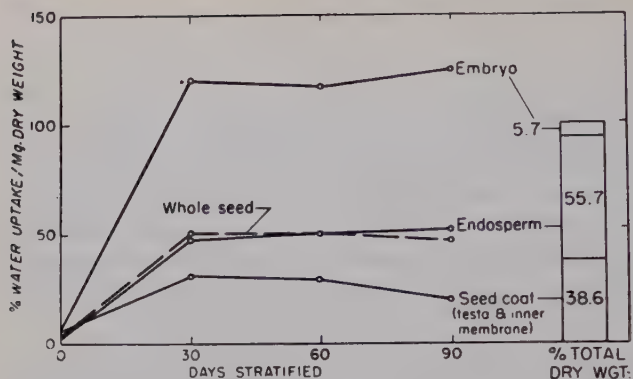


Figure 2. Percent of water absorbed in various organs of seeds of *P. lambertiana*.

to absorb water. Excised embryos and endosperm were killed by 30-minute exposure to 100°C . During this treatment loss of water from the tissues was minimized by placing the seeds in tightly stoppered vials with little air space. The dead tissues were then placed under germinating conditions for 72 hours and the water uptake followed. Figure 4 shows that the dead embryo tissue was able to imbibe a considerable amount of water, just as the inert seed coat did (Figure 2).

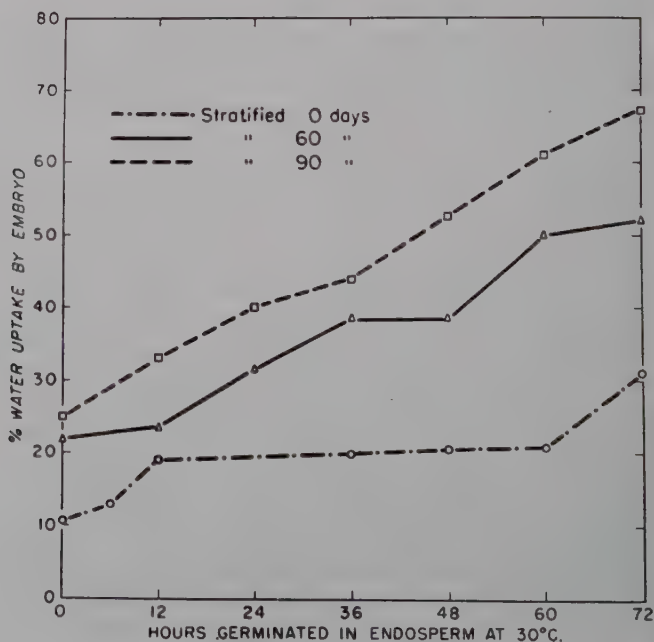
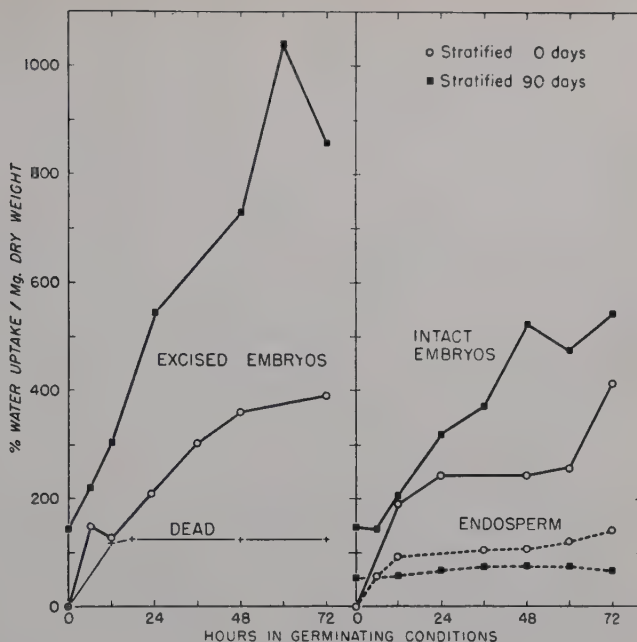


Figure 3. Rate of moisture absorption by embryos germinated in the endosperm (without testa or papery membrane) before and after stratification.

Figure 4. Moisture content of seeds of *P. lambertiana* germinated without the testa and papery membrane.



Time of germination. When seeds are first exposed to germinating conditions, imbibitional water uptake occurs. The seed moisture content then remains at a nearly level plateau for various periods of time. In this study the extent of this phase depended upon the seeds' prior treatment (Figure 3). The elapsed interval after which germination occurred at 30°C, as evidenced by radicle growth, appeared to be in part related to the moisture content of the embryo. Germination of unstratified seeds without the testa and inner membrane was first apparent after about 60 hours exposure to germination conditions; 30-day stratified seeds germinated after about 18 hours; 60-day stratified seeds germinated after about 8 hours; and the radicle of seeds stratified 90 days began elongating on exposure to growing temperatures, if the radicle was not already protruding after 3 months of stratification. In relating these observations to the curves in Figure 3, it appears that germination at 30°C occurs when the embryo contains approximately 23 percent of the seed moisture. This compares favorably to the 26 percent moisture content found essential for the germination of seeds of *Pinus thunbergii* at room temperature (Goo, 1952).

According to Goo (1951), the second active period of water uptake which accompanies the cytologically apparent growth, that time which we have designated as the beginning of germination, is the result of physiological

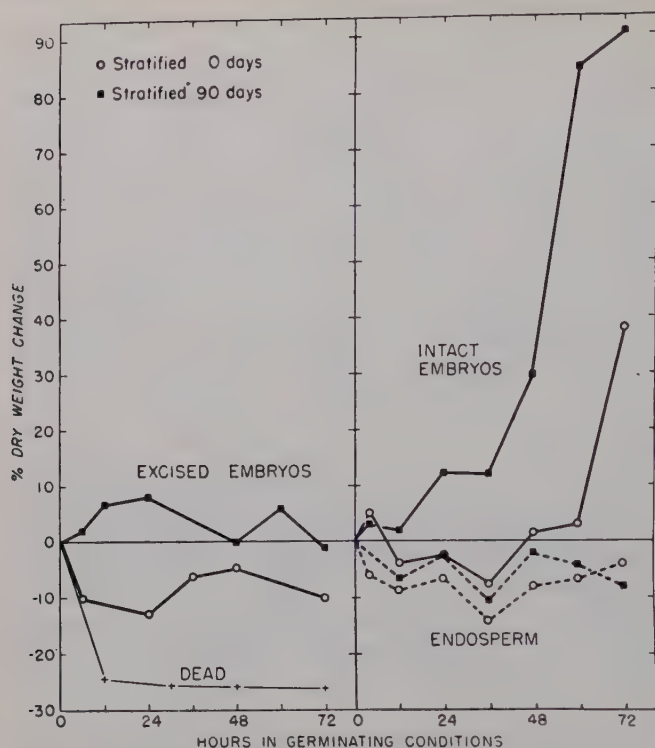


Figure 5. Dry weight changes in embryo and endosperm from seeds of *P. lambertiana* germinated without testa or papery membrane.

changes which occur while the embryo remains with the water content at near static plateau level. A comparison of Figure 3 with the curve for the water uptake of the dead embryo in Figure 4, illustrates what happens when a tissue is unable to go through the physiological stages which must precede germination. In such cases the embryo absorbs water to a given level and then no additional water is absorbed.

Studies indicating that a delay in germination may occur, although a seed has reached an apparent water saturation level, have been published for seeds of *Pinus thunbergii*, *P. densiflora*, *Larix kaempferi* and *P. jeffreyi* (Goo, 1951; Hatano, 1951; Stone 1957). However, variations and water uptake patterns of germinating seeds may be misleading unless concomitant dry weight changes of the germinating structures are also followed.

Dry weight changes. In seeds stratified 90 days a greater increase in dry weight occurred between 48–60 hours than during the 60–72 hour interval (Figure 5). However, Figure 4 indicates that the percent water uptake drops in the 48–60 hour period. Actually, the increase in dry weight, the base for computing the moisture content of the embryo, accounts for most of the decreased rate of moisture uptake of the embryo during that interval. Only

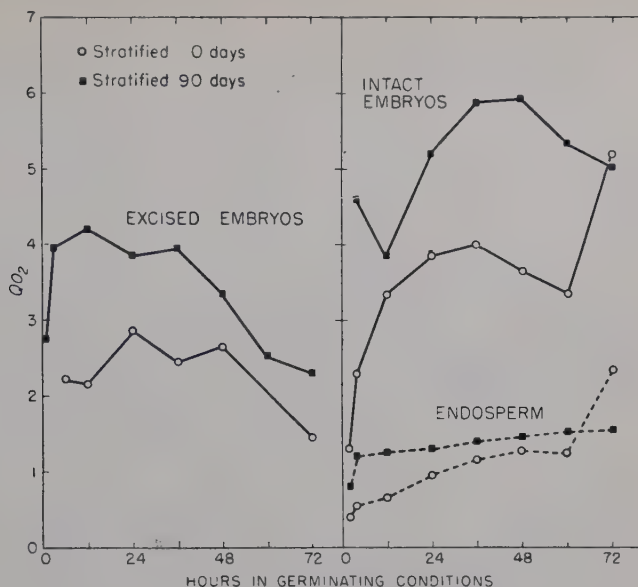


Figure 6. Oxygen uptake by the component parts of seeds of *P. lambertiana* germinating without testa or papery membrane.

weight increases during the 60–72 hour interval of embryos germinated intact were statistically significant at the 1 percent level.

It was noted (Figure 4) that the dead embryo imbibes moisture much as the inert seed coat does. The question of whether the dry weight of the dead embryo also changes was investigated. Figure 5 A shows that the dead tissue does lose weight. This characteristic has also been observed with dead seeds of barley, and the losses were attributed to a leaching out of soluble constituents (R. Brown, 1943 a). This hypothesis, although not tested in these studies, is probably applicable. Since heat destroys permeability barriers, more extensive leaching would be expected to occur from the dead embryos, and in particular after initial exposure to water, as was observed (Figure 5 A).

Oxygen uptake. The variations in oxygen uptake per mg dry weight, Q_{O_2} , which occur in germinating seeds of *P. lambertiana* are recorded in Figure 6. When the embryo was germinated in the endosperm, it reached a Q_{O_2} of 5.0 after 72 hours. When it was germinated for the same length of time outside the endosperm, the Q_{O_2} was considerably lower, that is, 1.5 to 2.5. Embryos from stratified seeds have a higher initial Q_{O_2} than non-stratified embryos. The observed values are comparable to those reported by Link and co-workers (1952) for tomato stem slices, and in other plant tissues by James (1953).

The endosperm also has an inherent respiratory activity, albeit lower than that of the embryo. The endosperm Q_{O_2} rose to about one half the Q_{O_2} of the germinating embryo at the end of 72 hours (Figure 6).

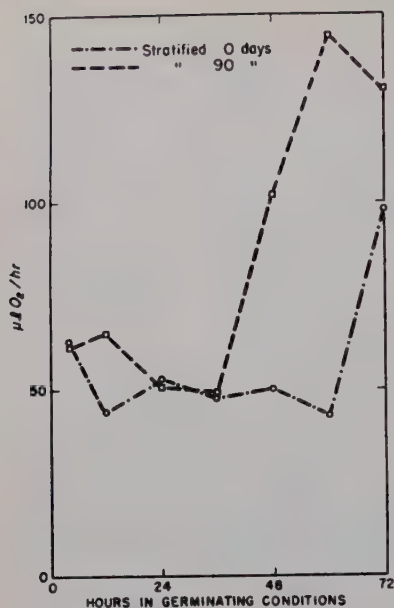


Figure 7. Changes in oxygen uptake in embryos germinated in the endosperm without testa or papery membrane.

Oxygen uptake is known to increase with the start of growth processes (James, 1953). Since water uptake appears to vary directly with germination activity and dry weight increases, in seeds germinated at 30°C, a sharp rise in oxygen uptake by the germinating embryo should occur at the point where rapid growth and rapid water uptake begin. A comparison of the curves in Figures 1 and 4B indicates that such a rise in oxygen consumption did occur at the time when increased water uptake began.

CO₂ and R.Q. measurements. Any measurement of CO₂ produced by whole tissues or cellular units, consequently any measurement of the respiratory quotient, R.Q., must be corrected for the CO₂ bound by the solutions and tissues (Umbreit *et al.*, 1949). Because some workers have corrected for bound CO₂ while others have not (Brown, J. W., 1939; Pack, 1921), preliminary experiments were performed to determine the most desirable procedure. Table 1 shows that a correction for bound CO₂ is not necessary since change in the amount of CO₂ bound by germinating embryos amounts to only 2 to 3 percent of the CO₂ evolved.

The R.Q. of both the embryo and endosperm of ungerminated seeds of *P. lambertiana* was initially between 0.98 and 1.2 (Figure 8). The fact that the R.Q. decreased more rapidly when the embryo was germinated outside the endosperm than when it is germinated intact indicated that the endosperm probably supplied substrates to the embryo early in the germination

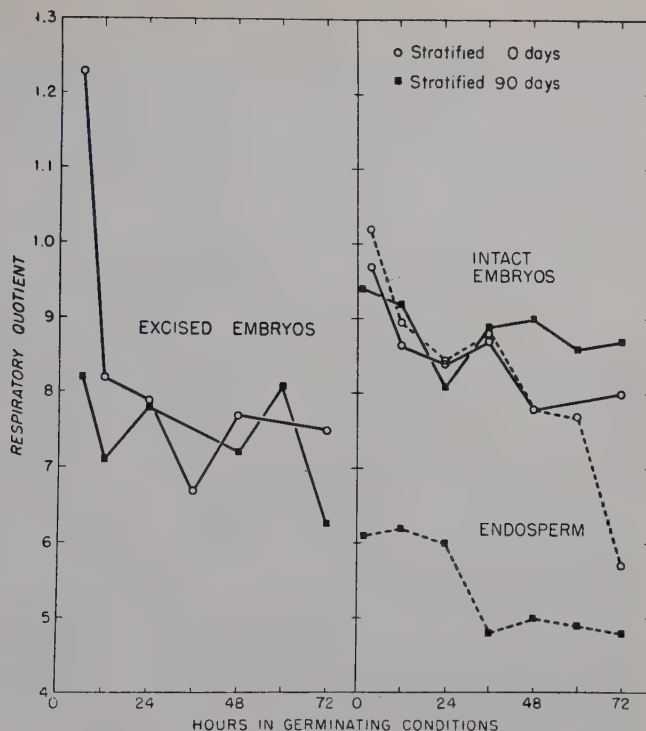


Figure 8. Respiratory quotient of germinating seeds of *P. lambertiana*.

process. The drop in R.Q. of the non-stratified embryo germinated in the endosperm paralleled that of the endosperm during the first 48 hours of germination; in both cases the R.Q. reached a value of around 0.80—0.85. Similar values were recorded by Stone (1948). In the next 24 hours the embryo remained at 0.8 while the endosperm dropped to less than 0.6. When seeds were stratified 30 days, the R.Q. of the endosperm was initially 0.6,

Table 1. Bound carbon dioxide, *Pinus lambertiana* embryos.

Germination time (hours)	Embryos germinated outside endosperm					
	Unstratified			Stratified 90 days		
	Bound $\mu\text{l}/\text{mg}$	Change in $\mu\text{l}/\text{hr}/\text{mg}$	Respiratory Q_{CO_2} $\mu\text{l}/\text{hr}/\text{mg}$	Bound $\mu\text{l}/\text{mg}$	Change in $\mu\text{l}/\text{hr}/\text{mg}$	Respiratory Q_{CO_2} $\mu\text{l}/\text{hr}/\text{mg}$
0	0.29	—	—	.81	—	—
12	0.41	+ 0.1	2.7	.46	- 0.3	3.0
24	0.62	+ 0.1	3.3	.71	+ 0.1	3.0
48	0.45	- 0.1	1.7	.29	- 0.1	2.3
72	0.40	- 0.04	1.8	.59	+ 0.1	1.8

and during germination dropped to 0.5. The endosperm of seeds stratified 90 days followed a similar pattern of respiratory changes (Figure 8). The R.Q. of all embryos germinated outside the endosperm dropped to a lower value at the end of 72 hours than did that of embryos germinated inside the endosperm.

Discussion

The germination pattern of unstratified and stratified seeds are similar although not identical. In unstratified seeds germinating at 30°C, a rapid uptake of water occurs during the first 12 hours, probably due to imbibitional forces (Crocker and Barton, 1953). From about 12 to 60 hours no further increase in absorbed water occurs. A low rate of oxygen uptake (Q_{O_2}) occurs during these first two phases, corresponding to the curve for water absorption. However, changes in the respiratory quotient during the second phase indicate that the enzymes of the embryo are active. Studies of mitochondrial particles from water saturated sugar pine seeds in these initial germination phases also indicate that the particulate oxidative enzymes are functioning (Stanley and Conn, 1957; Stanley, 1957).

It has been suggested that during the first two phases of germination, enzyme activities lead to the accumulation of compounds essential for cell division and the synthesis of protoplasm (Goo, 1951; Steinbauer, 1937). When certain threshold levels of these required substrates or moieties are reached, the third phase of germination starts. In this last phase growth and increase in dry weight occur, accompanied by the rapid uptake of oxygen and water. Results reported in this study may be interpreted as supporting this hypothesis.

In unstratified seeds the third phase of germination begins after about 60 hours. In stratified seeds the pattern of changes occurs more rapidly. When pretreated seeds are placed in germinating conditions, they appear to be at an advanced point on the germination-respiratory activity curves. Respiratory differences have also been reported between mitochondria extracted from stratified and unstratified seeds (Stanley and Conn, 1957).

A rapid increase in oxygen uptake, such as occurs at the beginning of the third phase, is also known to occur in ripening fruit at the beginning of the climacteric. During the climacteric rise, the storage products are converted to respirable substrates with a great increase in oxidative activity (Millerd *et al.*, 1953). It has been suggested that this sudden rise in oxidative activity is the result of the occurrence of an uncoupling agent in the phosphorylative mechanism. By this mechanism the limited amounts of adenylate

present in the fruit is no longer a barrier to the oxidation of the available substrates. Such a mechanism could conceivably be operative in germinating pine seeds.

Results of Jansson (1955) indicate that enzyme activity can not always be correlated with capacity for growth in seeds. It therefore appears that if the mechanism of germination in pine seeds is to be studied further then other procedures must be adopted. By germinating stratified and non-stratified seeds at lower temperatures (Stone, 1958), it may be possible to determine the nature of the accumulated intermediate or growth factor.

Summary

1. The changing patterns of water, dry weight, oxygen uptake and respiratory quotients during the first 72 hours of germination were followed for seeds of *Pinus lambertiana* from which the testa and inner papery membrane had been removed.
2. Water uptake by unstratified seeds, germinated at 30°C, followed three distinct phases: a rapid initial uptake, a period of little water uptake, and a third phase after about 60 hours, in which a renewed rapid uptake occurs.
3. The seed coat and endosperm of the germinating seed absorbed water to about 40 and 150 percent respectively. On the other hand the embryo absorbed water in proportion to the surface area and time exposed to moisture. The percent moisture per mg. dry weight, absorbed by the embryo was 10—15 times greater than that by either the endosperm or seed coat.
4. The oxygen uptake pattern generally followed that of water absorption. In the unstratified seed an upsurge occurred at the 60 hour interval; in stratified seed it occurred after 4—24 hours. The Q_{O_2} of embryos germinated inside the endosperm was found to reach a value of about 5 after 72 hours, regardless of the previous period of stratification. The Q_{O_2} of the embryo germinated outside the seed coat attained a value between 1.5 and 2.5 after 72 hours.
5. The respiratory quotient (R.Q.) of the embryo and endosperm of unstratified seed when initially set out to germinate was above 1. During the first 72 hours of germination the R.Q. of the embryo decreased to about 0.8, the endosperm decreased to about 0.6. The R.Q. of the embryo of 90 day stratified seed was initially about 0.9, that of the endosperm was 0.6. After 72 hours they had decreased to approximately 0.8 and 0.5 respectively.

6. The significance of the lag-period in germination — as a period in which substrate changes occur, or growth factors are produced, or a mechanism is developed for overcoming limiting amounts of phosphate acceptor — is discussed.

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Der primäre Angriffsort pflanzeneigener Hemmstoffe

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In der neutralen Fraktion ätherischer Extrakte aus Sprossen von Erbsenpflanzen befinden sich Hemmstoffe. Diese beeinflussen regulativ die korrelative Knospenhemmung, die Adventivwurzelbildung und das Wurzelwachstum (Libbert 1954, 1955 ab, 1956, 1957 ab). Wiederholt vermutete ich, daß einer dieser Hemmstoffe ein Antiauxin (=kompetitiver Auxinantagonist) sei (1955 a, 1956, 1957 b). Die Belege dafür lassen aber einen Beweis nicht zu.

Durch Kombinationsversuche von Hemmstoff mit Auxin lassen sich Rückschlüsse auf den Angriffspunkt von Hemmstoffen ziehen. Solche Versuche können die Antiauxin-Natur eines Hemmstoffes nicht beweisen, aber wahrscheinlich machen oder widerlegen. Es wurden Kombinationen von Hemmstoffextrakten (*Pisum*) mit Auxin (Indol-3-essigsäure=IES) an Segmenten aus *Pisum*-Internodien getestet. Solche Versuche können auf gestellte Fragen nur komplexe Antworten geben; denn die Möglichkeiten für einen Hemmstoff, den Auxin-Stoffwechsel zu beeinflussen, sind mannigfaltig.

Um eindeutigere Ergebnisse zu erhalten, wurden anhand von in-vitro-Versuchen 3 derartige Möglichkeiten untersucht: 1. Besteht ein direkter Einfluß der Hemmstoffe (nicht auf die Auxinwirkung, sondern) auf Auxin selbst? 2. Besteht ein Einfluß der Hemmstoffe auf IES-Oxydase? 3. Besteht ein Einfluß der Hemmstoffe auf das Enzymsystem, das die Entstehung von IES aus Tryptophan bewirkt?

In den untersuchten Extrakten kommen wenigstens 2 verschiedene Hemmstoffe vor (Libbert 1955 b). Trotzdem wurde hier der Gesamtextrakt unter-

sucht, um später (bei evtl. positiven Ergebnissen) die wirksamen Extrakte fraktioniert anzuwenden.

Gewinnung der neutralen, hemmstoffhaltigen Extrakte: Extraktion zerschnittener grüner Erbsensprosse (Sorte Senator) mit Äther bei $+1^{\circ}\text{C}$ über Nacht. Überführung in Wasser durch Vacuum-Destillation des Äthers. Alkalisierung der wäßrigen Lösung mit NaHCO_3 unter Schütteln. 3maliges Ausschütteln der nicht-sauren Bestandteile mit Äther. Überführen der ätherischen Lösung in soviel (dest.) Wasser bzw. Pufferlösung, wie dem 3. Teil des extrahierten Pflanzenmaterials entspricht; also Hemmstofflösung 3fach konzentriert ($=\text{HS } \frac{3}{1}$). — Übrige methodische Einzelheiten vgl. die einzelnen Abschnitte.

1. Kombinationsversuche IES-Hemmstoffextrakt

Testmethode: Erbsenpflanzen bei völliger Dunkelheit (27°C) aufgezogen. Aus den 3. Internodien (noch in Streckung) 7 mm lange Zylinder geschnitten; übertragen in Petrischalen mit 4 ml Testlösung. Sämtliche Handhabungen bei sehr schwachem Grünlicht; das war notwendig: Sonst reagierten die Zylinder unregelmäßig und schwächer auf Hemmstoff. Nach Versuchsende mikroskopische Längenmessung.

Die Testschalen enthielten 0,05 mol Phosphatpuffer, $\text{pH } 6,1$ (=optimaler pH -Wert). Die geringe Hemmung (jedenfalls bei längerer Versuchsdauer) durch die rel. hohe Pufferkonzentration wurde in Kauf genommen, da schwächere Puffer (0,02 mol) ungenügende Pufferkapazität hatten (pH -Drift in 16 h bis $\text{pH } 5,5!$). — Zucker wurde nicht zugesetzt, da er auch bei längerer Versuchszeit nicht förderte.

Der stündliche Zuwachs war ohne IES 12, mit IES (0,1—1 ppm) nur 3 Stunden lang konstant, um dann abzusinken. Die Versuchszeit betrug deshalb 3 Stunden. Wachstum ohne IES in 3 Std. etwa 0,75 mm, mit IES (0,2 ppm) etwa 1,4 mm (Abb. 1). Optimal etwa 10 ppm IES.

Hemmstoffhaltiger Extrakt gab unregelmäßige Ergebnisse, oft gar keine Hemmung. Das liegt am Vorkommen von Indol-3-acetaldehyd in der neutralen Fraktion (Larsen 1944, Libbert 1955 a), der während der Versuchszeit IES liefert. Deshalb wurde der Aldehyd mit Dimedon entfernt.

Neutrale Fraktion (ätherisch) im Vacuum überführt in 10 ml Phosphatpuffer (0,1 mol, $\text{pH } 5$), Zusatz von 2 ml gesättigter Dimedon-Lösung (Dimethyldihydroresorzin), geschüttelt und 20 Std. im Dunkeln stehen gelassen. 2mal mit Äther ausgeschüttelt; ätherische Lösung 3mal mit Wasser ausgeschüttelt. Trotz letzteren Ausschüttelns gelangen geringe Dimedonmengen in den ätherischen Hemmstoffextrakt. Deshalb wurden die Verdünnungsstufen des Hemmstoffes vor der Aldehyd-Entfernung hergestellt und alle gleicherweise (ebenso die Kontrolle: dest. Wasser) mit Dimedon behandelt. Es befanden sich demnach in allen Testlösungen gleiche, geringe Dimedonmengen, die das Wachstum der Testobjekte leicht hemmten.

Kombiniert wurden die IES-Konzentrationen 0; 0,02; 0,05; 0,1 und 0,2 ppm mit den Hemmstoffkonzentrationen 0; $\frac{1}{3}$ und $\frac{3}{1}$ (Abb. 1). Hemmstoffextrakt allein hemmt das Wachstum signifikant (Varianzanalyse).

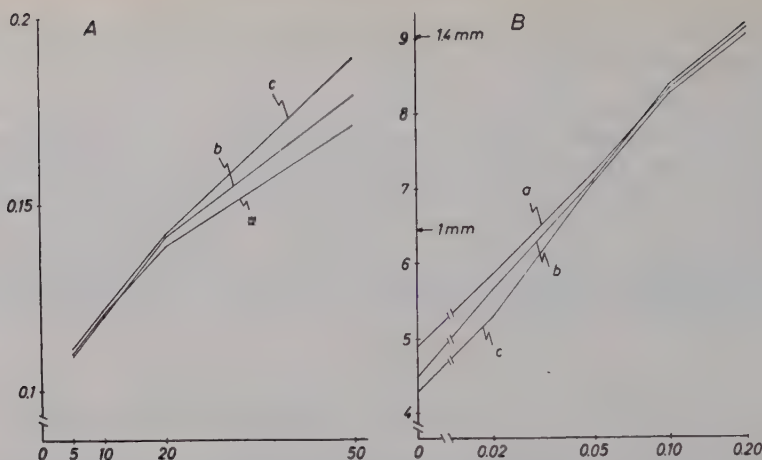


Abb. 1. Wirkung von IES/Hemmstoff-Kombinationen auf das Wachstum von *Pisum-Internodien-Zylindern*. Testdauer: 3 Std. Jeder Kurvenpunkt: Mittelwert aus je 60 Zylindern. — 1 A: Doppelreziproke Darstellung. Ordinate: Zuwachs in Okulareinheiten $^{-1}$ (OE^{-1}). Abszisse: IES-Konzentration in ppm^{-1} . — 1 B: Logarithmische Darstellung. Ordinate: Zuwachs in OE. Abszisse: IES-Konzentration in ppm (logarithmischer Maßstab). — 1 OE = 0,155 mm. a: ohne Hemmstoff; b: Hemmstoff $1/3$; c: Hemmstoff $2/3$.

Auxinkonzentrationen von 0,05 ppm aufwärts unterbinden jede Hemmstoffwirkung. Das in Abb. 1 B dargestellte Verhalten (Konvergenz der Kurven zu höheren IES-Konzentrationen) muß zwar bei Kombination eines Auxins mit einem Antiauxin erwartet werden. Aber die Hemmstoffwirkung ist (im Vergleich zu anderen Tests: Samenkeimung, Knospenwachstum u.a.; Libbert l.c.) so schwach und wird durch so geringe Auxinkonzentrationen aufgehoben, daß Zweifel am Antiauxincharakter der Hemmstoffe auftreten.

Auch die doppelreziproke Darstellungsweise (nach McRae und Bonner 1953) gibt kein eindeutiges Ergebnis (Abb. 1 A): Die Wirkungskurven sind keine Geraden, wie von diesen Autoren gefordert und für *Avena-Coleoptilzylinder* nachgewiesen. — Eigene Untersuchungen an *Triticum-Coleoptilzylindern* (allerdings längere Versuchszeit, 20 Std.: keine Proportionalität Zuwachs/Zeit) brachten für die Hemmstoffextrakte keine anderen Ergebnisse als die Versuche mit *Pisum-Zylindern*.

2. Beeinflusst der Hemmstoffextrakt IES in vitro?

IES (10 ppm) und Hemmstoff ($2/3$) standen gemeinsam 18 Std. in gepufferter Lösung (Phosphatpuffer 0,05 mol, pH 6,8; 10 ml) unter verschiedenen Bedingungen: Dunkelheit oder Licht (100 Watt-Lampe), offene Reaktions-

Tabelle 1. Wirkung von IES (10 ppm) und Hemmstoff ($^3/1$) im *Triticum-Coleoptilzylinder*-test nach 18stündigem gemeinsamen Stehen in gepuffelter Lösung und anschließender Trennung der beiden Komponenten. 3 Versuchsserien zu je 3 Versuchen. Zahlen: Zuwachs in mm, Mittelwerte aus je 60 Zylindern.

Bedingungen während des gemeinsamen Stehens von IES und Hemmstoff	Kontrollen			IES nach 18 h	Hemmstoff nach 18 h		Hemmstoff+IES nach 18 h	
	Aqua dest.	IES	Hemmstoff		Saure Frakt.	Neutr. Frakt.	Saure Frakt.	Neutr. Frakt.
Dunkel, verschlossen	4,7	8,7	3,9	8,7	4,7	4,1	8,7	4,1
Licht, verschlossen ...	3,9	7,9	3,5	7,6	4,2	3,5	7,5	3,7
Licht, offen	5,0	8,8	4,6	9,0	4,9	4,4	8,7	4,4
Mittelwerte	4,5	8,5	4,0	8,4	4,6	4,0	8,3	4,1

gefäße (Sauerstoffzutritt) oder geschlossene. Danach wurden in Anlehnung an Larsen (1955) die neutralen Hemmstoffe wieder vom sauren Auxin getrennt. Beide Fraktionen wurden mit *Triticum-Coleoptilzylindern* getestet.

Gemisch mit NaHCO_3 alkalisiert, wäßrige Lösung mehrfach mit Äther ausgeschüttelt: wäßriger Rest=1. saure Fraktion. Ätherische Lösung im Vacuum eingengt und mit NaHCO_3 ausgeschüttelt; ätherischer Rest=neutrale Fraktion, wäßriger Teil=2. saure Fraktion. Saure Fraktionen vereinigt und nach Larsen mit HCl gegen Methylorange titriert und mit Äther ausgeschüttelt; ätherische Lösung=saure Fraktion. Die ätherischen Lösungen im Vacuum in Phosphatpuffer überführt und getestet. Verwendet wurden 7 mm lange *Triticum-Coleoptilzylinder*, Testdauer 20 Std. — Nach der Trennung sind die neutrale Fraktion völlig, die 2. saure Fraktion fast auxinfrei (Vorversuche ohne Hemmstoff; IES allein) und beide sauren Fraktionen hemmstofffrei (Vorversuche ohne IES; Hemmstoff allein).

Die Ergebnisse (Tab. 1) waren unter allen Bedingungen die gleichen: Es ist keine in-vitro-Beeinflussung zwischen IES und Hemmstoff nachzuweisen. — Gleiche Ergebnisse wurden erhalten, wenn nach 18 Std. IES und Hemmstoff nicht voneinander getrennt wurden, sondern das Gemisch mit Salkowski-Reagenz gefärbt und danach der Auxingehalt kolorimetrisch festgestellt wurde: Die 18stündige Hemmstoffbehandlung beeinflußt die kolorimetrisch nachweisbare IES-Konzentration nicht.

3. Beeinflusst der Hemmstoffextrakt die IES-Oxydase in vitro?

IES-Oxydase („Erbsenenzym“) wurde in Anlehnung an Gordon und Sanchez-Nieva (1949) hergestellt. 30 g etiolierte Erbsensprosse zerrieben mit Quarzsand und 25 ml dest. Wasser; zentrifugiert; Satz verworfen; Eiweiß niedergeschlagen mit Azeton (10 : 4); erneut zentrifugiert; Bodensatz aufgenommen in Phosphatpuffer (0,05 mol, pH 6,8); erneut zentrifugiert; überstehende klare Lösung als Enzym verwendet. — 9,4 ml IES-Lösung (10 ppm) ohne oder mit Hemmstoffzusatz (gepuffert

Tabelle 2. *Kolorimetrisch (Salkowski-Test) erfaßbare IES-Konzentration nach Behandlung von 10 ppm IES mit IES-Oxydase \pm Hemmstoff. 3 Versuchsgruppen von je 10 Versuchen, Mittelwerte. Zahlen: Nachgewiesene IES-Konzentration in ppm.*

Hemmstoff-extrakt	Zusatz zur IES-Oxydase	Versuchsdauer	IES	IES + Oxydase	IES + Oxydase + HS 1/1	IES + Oxydase + HS 3/1
Nicht gekocht ...	—	7 Std.	9,3	7,8	7,4	7,4
Aufgekocht	—	7 Std.	9,9	8,6	8,8	8,9
Nicht gekocht ...	Mn ⁺⁺ ; DCP	1 Std.	9,9	4,7	—	5,0

auf p_H 6,8) + 0,6 ml Enzymlösung sind das Reaktionsgemisch. Nach unterschiedlichen Zeiten Reaktionsgemisch in 2 gleiche Teile geteilt; diesen je 10 ml Salkowski-Reagenz (50 ml 35 %ige $HClO_4$ + 1 ml 0,5 mol $FeCl_3$) oder nur $HClO_4$ zugesetzt; beide Proben bei 530 m μ gegeneinander kolorimetriert (Metallinterferenzfilter; Halbwertsbreite 4,5 m μ).

Das Enzym oxydierte IES in 18 Std. völlig, bei Zusatz von $MnCl_2 + 2,4$ -Dichlorphenol (DCP) in 3 Std. Um eine evtl. Beschleunigung des Enzyms durch Hemmstoffextrakt feststellen zu können, wurden die Versuche abgebrochen, wenn (ohne Hemmstoff) eine teilweise IES-Oxydation stattgefunden hatte (Tab. 2). Ergebnis: Es besteht kein Einfluß von Hemmstoffextrakt auf die IES-Oxydase (varianzanalytische Auswertung ebenso wie im nächsten Abschnitt). Gleiche Ergebnisse wurden bei noch kürzerer Versuchsdauer erzielt und auch dann, wenn nicht die neutrale Fraktion des Hemmstoffextraktes verwendet wurde, sondern der Hemmstoff-Rohextrakt.

4. Beeinflusst der Hemmstoffextrakt das IES-bildende Enzymsystem in vitro?

IES entsteht in der Pflanze enzymatisch aus Tryptophan. Über die Intermediärprodukte ist nichts Endgültiges bekannt. Ich verwendete einen Enzymextrakt aus Sprossen grüner Erbsenpflanzen, der die Entstehung von IES aus Tryptophan katalysiert. Der Enzymextrakt (hier kurz „Tryptophan-Enzym“ genannt) besitzt sehr geringe IES-Oxydase-Aktivität; über seine Eigenschaften soll an anderer Stelle berichtet werden.

15 g grüne Erbsensprosse mit Quarzsand zerrieben in 12,5 ml Phosphatpuffer (0,05 mol, p_H 6,8); zentrifugiert; überstehende klare Lösung als Enzym verwendet. — 4,7 ml Tryptophan-Lösung (583 ppm = äquimolar IES 500 ppm) in gleichem Puffer, ohne oder mit Hemmstoffzusatz, + 0,3 ml Enzymlösung sind das Reaktionsgemisch. DL-Tryptophan zweierlei Herkunft wurde verwendet: Das zuerst benutzte (Feinchemie Berlin-Schöneberg) war mit 2 Salkowski-positiven Substanzen (von denen eine auch als reguläres Intermediärprodukt der enzymatischen IES-Bildung aus Tryptophan auftritt) in geringer Menge verunreinigt. Das später benutzte (Merck,

Tabelle 3. *Einfluß von Hemmstoffextrakt auf die Wirksamkeit des Tryptophan-Enzyms im 17stündigen Versuch.* Mittelwerte aus 2 Versuchsgruppen zu je 12 Versuchen. Zahlen: Extinktionen. Versuchsgruppe 1: Tryptophan Schöneberg; Versuchsgruppe 2: Tryptophan Merck.

Versuchs- gruppe	Extinktion bei: m μ	Trypto- phan	Trypto- phan + Enzym	Tryptophan + Enzym + HS 1/1	Tryptophan + Enzym + HS 3/1	LSD _{0,05}	LSD _{0,002}
1	530	—	0,30	0,26	0,16	0,06	0,09
1	465	—	0,19	0,18	0,13	0,03	0,06
2	530	0,04	0,22	—	0,14	0,02	0,04
2	465	0,12	0,15	—	0,12	0,02	0,04
Salkowski-Färbung		Gelb	Rot	Rot	Rot	—	—

Darmstadt) enthielt keine nachweisbaren Verunreinigungen. — Tryptophan färbt sich mit Salkowski-Reagenz gelb. Deshalb wurde nach Enzymeinwirkung außer bei 530 m μ auch bei 465 m μ (Metallinterferenzfilter; Halbwertsbreite 12,7 m μ) kolorimetriert; bei Tryptophan ist die Extinktion bei 465 m μ größer als bei 530 (im Gegensatz zu IES).

Im Reaktionsgemisch treten außer Tryptophan und IES noch andere Salkowski-positive Substanzen auf (papierchromatographischer Nachweis). Deshalb wurden die gemessenen Extinktionen nicht in IES-Konzentrationen umgerechnet. Tab. 3 enthält die Extinktionen. Ergebnis: Der Hemmstoffextrakt hemmt die Entstehung von IES aus Tryptophan signifikant.

Die „letzte signifikante Differenz“ (LSD) wurde varianzanalytisch berechnet aus der Fehlervarianz: Behandlung \times Wiederholung. Der Varianzanalyse wurden zuerst die Kolorimeterausschlagswerte zugrundegelegt. Nachdem sich im Barlett-Test herausgestellt hatte, daß die Kolorimeterausschlagswerte zwar meist, die Extinktionen aber stets homogene Varianzen lieferten, wurden später die Extinktionen varianzanalytisch verrechnet. Für die 4 Spalten der Tab. 3 ergibt der Bartlett-Test folgende P-Werte: 0,54; 0,70; 0,33; 0,69; also homogene Varianzen.

Die Komponente, deren Entstehung aus Tryptophan durch den Hemmstoffextrakt gehemmt wird, ist tatsächlich IES, nicht ein anderes Salkowski-positives Produkt: Nach 6stündiger Einwirkung von Enzym auf Tryptophan ließ sich papierchromatographisch im Reaktionsgemisch ohne Hemmstoff in allen derartigen Versuchen IES nachweisen; mit Hemmstoff dagegen nur in einem einzigen Versuch. Darüber wird später berichtet.

Wirksam ist der Hemmstoffextrakt selbst, nicht etwaige bei der Aufarbeitung erhaltene Verunreinigungen (Leerversuche); besonders auch nicht etwaige im Reaktionsgemisch verbliebene Spuren von Äther: Selbst Zusatz einiger Tropfen (!) Äther zum Reaktionsgemisch beeinträchtigte nicht die Wirksamkeit des Tryptophan-Enzyms.

5. Diskussion

Ein nativer Hemmstoff beeinflußt die enzymatische Auxinbildung *in vitro*. Dieser Befund kann physiologische Bedeutung haben, vorausgesetzt, daß dieselbe Beeinflussung auch *in vivo* stattfindet. Dafür fehlt noch der Beweis. Aber der Befund, daß die untersuchten Hemmstoffextrakte die Samenkeimung und das Knospenaustreiben (Prozesse, die mit Auxinsynthese verbunden sind) viel stärker hemmen als die Streckung von Sproßzylindern (ein Prozess, der höchstens unter sehr geringer Auxinsynthese abläuft), spricht für die Übertragbarkeit der *in vitro* gefundenen Ergebnisse auf die Verhältnisse *in vivo*. Nach papierchromatographischer Aufarbeitung der Hemmstoffextrakte lassen sich auf dem Papier mit Zylinder-Streckungstests keine Hemmstoffe nachweisen, gut dagegen mit Keimungstests (Liebenow).

Die Ergebnisse der Kombinationsversuche (Abschnitt 1) lassen sich mit den *in vitro* erhaltenen Befunden interpretieren: Wenn in den *Pisum*-Zylindern geringe Auxinsynthese während der Versuchszeit ablief, muß eine Substanz, die diese Synthese hemmt, die Streckung leicht hemmen. Geringe Auxinkonzentrationen müssen diese Hemmung aufheben. Diese Verhältnisse wurden gefunden (Abschnitt 1).

Der primäre Angriffsort des wirksamen Hemmstoffes ist nicht letztlich lokalisiert: Es muß festgestellt werden, in welchen Teilschritt der Auxinbildung aus Tryptophan der Hemmstoff eingreift. Die Kenntnis der nachweisbaren Zwischenprodukte der Reaktionskette $\text{IES} \rightarrow \text{Tryptophan}$ muß der Beantwortung dieser Frage vorausgehen. Entsprechende Untersuchungen sind in Arbeit.

Da in den verwendeten Hemmstoffextrakten mindestens 2 Hemmstoffe vorkommen (darunter die Substanz für die korrelative Hemmung), ist von Bedeutung, welcher derselben die *in vitro* wirksame Komponente ist. Da mehrere chemische und physikalische Eigenschaften der beiden Hemmstoffe bekannt sind (Libbert 1955 b), ist die Lösung dieses Problems nur eine Zeitfrage. Sollte sich herausstellen, daß die wirksame Komponente der Korrelationshemmstoff ist, dann ergeben sich wichtige Konsequenzen für den Mechanismus der korrelativen Hemmung. Diese zu erörtern, ist an dieser Stelle verfrüht.

Die negativen Ergebnisse der Abschnitte 2 und 3 verlangen keine besondere Interpretation. Vor 2 Jahren (Libbert 1955 c) wurde zwar eine Beziehung *in vitro* zwischen Auxin und einer Komponente der Hemmstoffextrakte gefunden. Dazu war aber eine extrem hohe IES-Konzentration notwendig (100 ppm); und trotzdem blieb der Auxin-Einfluß auf den Hemmstoff (trotz empfindlicherem Test) sehr schwach.

Summary

Extracts of pea plants containing the substance for correlative inhibition inhibit the enzymatical generation of indole-3-acetic acid (IAA) from tryptophane *in vitro*.

Experiments on the combined effects of various concentrations of IAA and inhibitor extract on elongation of pea stem sections did not decide whether or not there is an antiauxin in the extracts. There is only little influence of inhibitor extract on section elongation.

There is no influence of inhibitor extract on IAA-oxidase *in vitro*. A direct (non-enzymatical) relation between IAA and inhibitor extract could not be proved.

An der Durchführung der Untersuchungen waren Frl. H. Rummler, Frl. E. Schmidt sowie besonders Frl. Ch. Herbst beteiligt, wofür ich herzlich danke.

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The Influence of Water Deficit on Transpiration

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Introduction

Among internal factors influencing the transpiration of plants first importance must be given to those determined by the state of the water economy of the transpiring leaf. These are, for instance: the water-saturation of the leaf tissues, the supply of water to the transpiring surfaces, the changing hydration and permeability of the cells and cell parts on the path of this centrifugal diffusion of water through the leaf mesophyll, and the hydration and permeability of the transpiring surfaces proper.

Opinions as to the *quantitative* effect of these factors on transpiration have not hitherto been uniform. With regard to cuticular transpiration, it is considered today that the dehydration of the epidermal cell walls and particularly of the cuticle has a decisive effect on the intensity of cuticular transpiration (Härtel 1947), even if there is now some doubt as to possible irreversibility of cuticular dehydration such as was found by Gäumann and Jaag (1936). Main emphasis is placed on the static component of this action, that is the decrease of the diffusion pressure of water and the resulting reduction in tension of water vapour at the surface of the cuticle.

With regard to stomatal transpiration, there is doubt about "incipient drying" and its influence on transpiration (Bange 1953, Stålfelt 1956, Milthorpe and Spencer 1957). The calculations of the reduction of water vapour tension on the surface of the intercellular cell walls owing to an increase of their osmotic pressure (Renner 1915, Stålfelt 1956), however, take into account only the static effect of water deficiency. They further assume that

a completely unrestricted flow of water will make the levelling of the DPD across the whole section to the outer membrane of the intercellular cells possible, and therefore under conditions of loss of turgor its equilibrium with the osmotic pressure of the cell sap. In view of the fact that there are very probably diffusion pressure gradients inside these cell walls, the dynamic factor of the effect of the water deficit on stomatal transpiration must be taken into account. Klemm (1956) in his work on the transpiration resistance of the cell walls of the mesophyll shows as a result of experiments on leaves with the epidermis removed that the transpiration resistance of mesophyll cell walls, which increases rapidly with decreasing relative humidity of the air, is an important regulator of the intensity of stomatal transpiration. This is so particularly because it may be assumed, according to the author's model experiments, that air in the intercellular spaces is far from being saturated with water vapour. Mainly experimental difficulties, particularly the difficulty of measuring the small stomatal apertures *in situ*, have prevented any proof of this influence. Hygen (1951), on the basis of pioneer work by Fukuda (1935), which has been generally underestimated, developed a quantitative analysis of these transpiration (loss) curves of excised leaves with success. This analysis, using a simple calculation, makes it possible to compare the intensity of stomatal and cuticular transpiration with the values of the initial water deficit.

This analysis of the transpiration curves has been used in the present work, the aim of which is to contribute to the solution of the still open question as to the influence of the quantity and quality of water deficit in transpiring leaves on the intensity of both stomatal and cuticular transpiration.

Methods

Our three experiments (A, B, C) were made with sugar-beet leaves (*Beta vulgaris* ssp. *esculenta* (Salisb.) Gürke var. *altissima* Roessig, Dobrovická A variety). Leaves were selected from the central part of the leaf spiral, since these proved to be fairly identical as regards the main aspects of their water economy. In all experiments the excised leaves were first artificially saturated. Then in each variation of the experiment a certain water deficit was attained in the leaves, the petioles being constantly immersed in water, under controlled external conditions, that is in the first place higher or lower air humidity and the presence or absence of artificial ventilation. In view of the fact that the excised leaves — originally fully water saturated — had their petioles constantly immersed in water before the actual determination of the transpiration curves, *i.e.* before weighing was started, the required experimental deficit could be attained without interruption of the water supply. The water deficit arose in the leaves under conditions resembling natural conditions, that is it was due to the excess of transpiration over the uninterrupted

but insufficient water supply to the leaves at the time. Transpiration curves were determined by weighing the leaf blades (average initial weight about 15 g.) with an accuracy of 0.02 g. in five, later at ten-minute intervals, under fairly constant conditions (changes in relative air humidity $\pm 2\%$, changes in air temperature maximum $\pm 1^\circ\text{C}$, illumination 8–10,000 Lux). The water deficit of the leaves was expressed as the weight at the time of full saturation at the start of the experiment and controlled at the end (in per thousand of fresh weight). The period over which transpiration curves were determined was 5 to 6 hours. When calculating the results we found that the correction of the values to standard relative humidity and atmospheric pressure had, under our experimental conditions, practically no effect on the quantitative characteristics of the curves obtained. The important requirement that the whole experiment, *i.e.* all experimental series, should be carried out under absolutely identical conditions was always strictly complied with.

The experimental results were mathematically treated in the main by Hygen's method (1951, 1953). From the values $r = -1000 \frac{d(\log G)}{dt}$ in $\text{mg. g}^{-1} \cdot \text{min}^{-1}$ the following calculations were then made for both phases: the intensity of total transpiration (*i.e.* stomatal and cuticular) at the start of the transpiration curve for zero water deficit

$$T_{(s+c)0} = \frac{r_{s+c}}{\log e} \cdot \frac{1000 - K}{1000 - (d + K)} \quad (1)$$

where K = dry weight of the leaf and d = deficit at the start of the transpiration curve in per thousand; further, the intensity of cuticular transpiration, also at the start of the transpiration curve for zero water deficit

$$T_{c0} = \frac{r_c}{\log e} \cdot \frac{G_c}{1000} \cdot \frac{1000 - K}{1000 - (d + d_c + K)} \quad (2)$$

where G_c = weight of the leaf at the beginning of the cuticular phase reduced to the initial weight of 1000; read from the graph d_c = loss of water at the start of the cuticular phase of the curve in per thousand of the initial weight.

The correlation coefficients and regression coefficients and their statistical significance were calculated by the usual method and the usual *t*-test.

Results

The aim of this work is, as stated above, to contribute to the solution of the question of how a water deficit affects the regulation of transpiration intensity when acting through internal factors not associated with regulation of the stomatal aperture. Therefore, attention was paid to those phases of the loss curves where it can be assumed that stomatal changes are not exerting any influence: the stomatal phase and the cuticular phase which in a relatively simple form can reflect the influence of the hydration state on transpiration.

The exponential character of the curves showing the relation of transpira-

tion intensity to the development of the total water deficit in both phases of the curve without changes in stomatal aperture (Fukuda's fundamental equation, 1935: $\frac{dWt}{dt} = -kW_0e^{-kt}$) is itself proof of the logical influence of water deficit on transpiration intensity. It is also necessary to note that a water deficit, which has developed under certain experimental conditions in the course of the determination of curves of transpiration loss, does not act in a similar way to an equally high deficit existing from the beginning and perhaps developing under different conditions. This assertion will be evident from the experimental data. The course of the individual transpiration curves itself does not provide numerical data for observing the influence of the quantity and quality of water deficit on transpiration intensity. For this purpose we must use transpiration curves of leaves having initial deficits of various magnitude and developed under various conditions (see Methods).

In the experiments transpiration curves of leaves with quantitatively and qualitatively varying initial water deficits (*i.e.* of varying magnitude and developed at varying rates) were analysed. The values obtained for both exponential phases of the transpiration curves were reduced to the zero initial deficit. The values for the intensity of stomatal and cuticular transpiration thus calculated were used to obtain the correlation coefficients between these intensities and the quality and quantity of the initial deficit. The difference between the individual calculated values of transpiration intensity would themselves be only an expression of individual variability according to two assumptions which were made in the detailed analysis of transpiration curves carried out by Hygen (1951). The first assumption is that the evaporation component of transpiration is proportional to the total "concentration" of free water in the transpiring organ (*i.e.* with a constant volume of its content). From this assumption follow equations for reducing the transpiration intensity, obtained from graphs of the phases of transpiration curves with which we are concerned, to the initial values, *i.e.* for example, to values corresponding to zero water deficit with maximum turgor. The second assumption is that the concentration of water molecules in the surface layers from which transpiration takes place (surface of the cuticle, outer surfaces of cell walls limiting intercellular spaces) is proportional to the total water concentration in the transpiring leaf. If these assumptions are considered to be valid, then the individual differences in the calculated intensities of stomatal and cuticular transpiration must be caused, as we have already pointed out, solely by individual variations in the leaves. In this case we could not expect to find any significant correlation between the transpiration rates and the initial water deficit either as regards its quality or its quantity. A significant correlation must, therefore, be considered as an

indication of the influence of water deficit on transpiration intensity and an explanation must be sought.

Cuticular Transpiration

Table 1 gives numerical results of cuticular transpiration from the transpiration curves of sugar-beet leaves in experiment A. In the fourth column is the calculation of the intensity of the initial cuticular transpiration with

Table 1. Numerical data of cuticular and stomatal transpiration intensity and their reduction to zero water deficit in experiment A. Transpiration values in $\text{mg.g}^{-1} \cdot \text{min}^{-1}$ on fresh weight basis.

No.	Initial water deficit in per thousand d	The rate of the increase of initial deficit in per mille/hour d_h	Cuticular transpiration intensity, reduced to zero deficit ICT	Total transpiration intensity, reduced to the start of the curve	Total transpiration intensity, reduced to zero water deficit IT	Stomatal transpiration intensity, reduced to zero water deficit IST
1	25	25	0.37	1.22	1.25	0.88
2	46	46	0.33	1.08	1.14	0.81
3	46	46	0.53	1.08	1.14	0.61
4	44	44	0.46	0.82	0.88	0.41
5	49	49	0.48	1.11	1.17	0.70
6	18	18	0.35	1.22	1.24	0.89
7	4	4	0.47	1.22	1.22	0.75
8	63	63	0.28	0.76	0.82	0.54
9	46	46	0.40	0.82	0.87	0.47
10	39	39	0.44	1.04	1.08	0.64
11	62	62	0.33	1.02	1.13	0.80
12	36	36	0.35	1.00	1.03	0.69
13	53	53	0.37	0.64	0.68	0.31
14	43	43	0.40	0.89	0.93	0.54
15	86	57	0.38	0.95	0.99	0.59
16	93	62	0.25	0.90	0.99	0.75
17	38	13	0.41	1.23	0.28	0.87
18	29	10	0.34	0.99	1.03	0.68
19	38	13	0.33	1.13	1.18	0.85
20	58	29	0.38	0.99	1.07	0.69
21	66	33	0.33	1.08	1.17	0.84
22	8.6	4.3	0.60	1.77	1.81	1.21
23	16	8	0.71	1.68	1.71	0.99
24	27	13.5	0.51	1.77	1.83	1.32
25	7	3.5	0.51	1.38	1.40	0.89
26	10	5	0.58	1.29	1.31	0.72
27	69	34.5	0.63	1.02	1.10	0.48
28	15	8	0.58	1.29	1.32	0.74
29	98	49	0.42	1.32	1.52	1.10
30	30	15	0.51	1.29	1.45	0.85
31	72	36	0.51	1.40	1.52	1.10
32	28	14	0.67	1.77	1.84	1.17
33	36	18	0.55	1.58	1.65	1.10
34	20	10	0.56	1.22	1.25	0.69
35	28	14	0.52	1.38	1.48	0.95
36	26	13	0.49	1.18	1.22	0.73

Table 2. *Statistical evolution of the experiments and experimental conditions.* Correlation and regression coefficients between *cuticular* transpiration intensity and initial water deficit (indexes ICT/d) and rate of increase of initial water deficit (indexes ICT/d_h).

Experiment	A	B	C	Average
Experimental conditions:				
Relative air humidity.....	65 ± 2 %	62 ± 2 %	72 ± 2 %	66 ± 2 %
Air temperature	21 ± 1°C	22 ± 1°C	22 ± 1°C	21 ± 1°C
Illumination	5—6,000 Lx	5—6,000 Lx	5—6,000 Lx	5—6,000 Lx
n	36	36	48	—
N	34	34	46	—
Min. significant r when P = 0.01...	0.43	0.43	0.43	—
Min. significant r when P = 0.05...	0.33	0.33	0.29	—
Cuticular transpiration:				
Average cuticular transpiration intensity reduced to zero water deficit	0.45	0.48	0.45	0.46
r _{ICT/d}	— 0.48	— 0.57	— 0.41	— 0.49
b _{yICT/d}	— 0.026	— 0.035	— 0.021	— 0.027
r _{ICT/d_h}	— 0.57	— 0.71	— 0.49	— 0.59
b _{yICT/d_h}	— 0.039	— 0.031	— 0.032	— 0.034

zero water deficit. Between this value (ICT=intensity of cuticular transpiration) and the size of the initial total water deficit of the leaf (d), or the rate of increase of this deficit (d_h), correlation coefficients and regression coefficients are calculated for the whole complex of each simultaneously executed experiment. They are given in Table 2. Thus, for example, in experiment A the correlation coefficient between the intensity of cuticular transpiration, reduced to zero water deficit, and the level of the initial total water deficit of the leaf $r_{\text{ICT/d}} = -0.48$ was calculated. This correlation coefficient is very significant ($P < 0.01$).

While calculating the correlation coefficients between the intensity of cuticular transpiration, reduced to zero water deficit, and the rate of increase of the original water deficit (d_h, i.e. total water deficit in per cent per hour) it was found that this correlation coefficient was in all experiments markedly higher. Thus, in experiment A the correlation coefficient was $r_{\text{ICT/d}_h} = -0.57$, in experiment B -0.71 and in experiment C -0.49 . (In view of the absolutely low values of the correlation coefficients differences between the corresponding pairs are not statistically significant).

From the data given it is possible to draw the following conclusions at present: the existence of statistically very significant negative correlation coefficients between the values for the intensity of cuticular transpiration,

reduced to zero water deficit, and the initial water deficit is proof of a decrease of intensity of cuticular transpiration with rising total water deficit. This conclusion is valid not only for the influence of water deficit during loss of water from excised leaves without any water supply (as is indicated by the actual course of the transpiration curve in the cuticular phase), but also according to our experiments for water deficit originating in leaves with an available water supply (*viz.* description of method). This conclusion is valid, therefore, for conditions very similar to, qualitatively almost identical with, the normal origin of a natural daily water deficit in leaves on a plant.

At the same time in each experiment an even higher and equally significant correlation coefficient was found between the intensity of cuticular transpiration and the rate of increase of the total water deficit (r_{ICT/d_h} in Table 2). As has been stated already, the differences between the corresponding pairs of coefficients $r_{ICT/d}$ and r_{ICT/d_h} of the same experiment are not statistically significant. Nevertheless the very fact that in all experiments the second coefficient was markedly higher than the equally statistically significant first coefficient indicates that cuticular transpiration is not only directly influenced by the quantity of the total water deficit, but also primarily by its quality, expressed here as its rate of increase. The effect on the intensity of cuticular transpiration of a water deficit which develops slowly, *i.e.* over a longer period of time, is different to that of an equally large deficit which develops over a shorter period of time. Regression coefficients (see Table 2) $b_{y(ICT/d)}$ of the relation of the intensity of cuticular transpiration to the rate of water deficit (within our limits 0 to 10 % water deficit) varied from -0.021 to -0.035 $\text{mg. g.}^{-1} \cdot \text{min.}^{-1}$ for 1 % water deficit, which with a medium intensity of cuticular transpiration in our experimental conditions of 0.464 $\text{mg. g.}^{-1} \cdot \text{min.}^{-1}$ amounted to a reduction of transpiration intensity by an average of 6 % for 1 % water deficit. Analogous regression coefficients of the relation of cuticular transpiration to the rate of increase of water deficit [$b_{y(ICT/d_h)}$] were in our three experimental series -0.031 to -0.039 (average -0.034) $\text{mg. g.}^{-1} \cdot \text{min.}^{-1}$ for 1 % water deficit per hour. With the stated average initial intensity of cuticular transpiration of fully saturated leaves the reduction amounted to about 7 % for each rise of 1 % per hour in the rate of increase of water deficit.

Stomatal transpiration

As in the case of cuticular transpiration the correlation coefficients between the calculated values for pure stomatal transpiration with zero initial water deficit (IST) and the values of initial deficit (d), or the rate of its increase (d_h), were computed (Table 3).

Table 3. *The same as Table 2 but stomatal transpiration intensity.*

Experiment	A	B	C	Average
Average Stomatal transpiration intensity, reduced to zero water deficit	0.78	0.82	—	0.80
$r_{IST/d}$	— 0.25	— 0.27	—	— 0.82
$b_{yIST/d}$	— 0.024	— 0.026	—	— 0.025
r_{IST/d_h}	— 0.56	— 0.45	—	— 0.50
b_{yIST/d_h}	— 0.064	— 0.050	—	— 0.057

As in the case of cuticular transpiration it was shown that the differences between the individual values obtained for pure stomatal transpiration are not only due to individual variations, but also that it is possible to find very significant negative correlation coefficients between them and the quality of the initial deficit. Here also it was found that the coefficients between intensity of pure stomatal transpiration and the rate of increase of the initial deficit are particularly significant. These coefficients r_{IST/d_h} were always highly significant and higher than the negative correlation coefficients between the intensity of pure stomatal transpiration and the actual magnitude of the initial deficit ($r_{IST/d}$), which were not significant. (For experiments A and B see Table 3).

The very existence of highly significant correlation coefficients, particularly between the intensity of pure stomatal transpiration and the rate of increase of the initial water deficit, indicates a dependence of the intensity of stomatal transpiration on water deficit, particularly on its quality, *i.e.* on the rate of its increase. (The rate being expressed as the magnitude of water deficit per hour). Further, the fact that not only the coefficient r_{IST/d_h} is very significant in all experiments and is higher than $r_{IST/d}$, but that also the coefficient $r_{IST/d}$ was not significant in our experiments, indicates that the influence of water deficit on stomatal transpiration is substantially bound to the rate of increase of water deficit (see discussion). The lack of significance of the coefficient $r_{IST/d}$ is not in itself — as is known — a proof of the non-existence of linear relation.

Regression coefficients of the relation of the intensity of stomatal transpiration to the rate of water deficit $b_{yIST/d}$ are given in Table 3. Their values indicate that with an average initial intensity of stomatal transpiration of 0.78 or 0.82 mg. g.⁻¹ · min.⁻¹ under our experimental conditions the reduction in intensity of stomatal transpiration amounted to 3 % for 1 % of water deficit. Similar regression coefficients for the relation of the intensity of

stomatal transpiration to the rate of increase of water deficit b_{yIST/d_h} are also given in Table 3. For the initial intensity of stomatal transpiration as stated, the reduction of intensity of stomatal transpiration amounts to an average of 7 % for each 1 % of the rate of increase of water deficit per hour.

Discussion

The effect of the consequences of water deficit in controlling transpiration is generally accepted in the case of cuticular transpiration. Usually the static aspect is emphasised in conceptions of the mechanism of this control, *i.e.* reduction of hydrature, hydration of the cuticle itself, in particular its surface, and as a result the reduction in water vapour tension on the surface (Sresnewski, 1905 (cited according to Maximov, 1929), Shreve, 1931). The conception of the dynamic aspect of the mechanism is also undoubtedly logical; it is *de facto* the cause of the static effect. With a high transpiration rate and insufficiently rapid diffusion of water to the cuticle, dehydration of the cuticle occurs. The capacity for dehydration is linked with the presence of suberin, and, therefore, the phenomenon usually called "incipient drying" is accounted for by the state in the cuticle when dehydration decreases not only the resulting DPD of water and water vapour tension, but also the permeability of the cuticle to water. This reduction in permeability again, with continued transpiration, increases the dehydration of the cuticle, this then reduces permeability again, water supply slows down and so on. In complete agreement with these dynamic conceptions is the existence not only of correlation between cuticular transpiration and water deficit, but also a still stronger correlation between the intensity of cuticular transpiration and the rate of increase of water deficit. At the same time, the existence of this difference between the degree of the two correlations indicates the divergent effect of the total water deficit of a leaf according to whether the same deficit originated rapidly or slowly. It follows from this quite naturally that the value of the total water deficit differs from the deficit in peripheral parts, which is of course considerably higher. The difference in the strength of the correlation between the intensity of cuticular transpiration and the total water deficit and the rate of increase of this deficit is an indication of the significance of this dynamic component for the explanation of the phenomenon described. It is an expression of the existence of a marked diffusion gradient within the leaf. This gradient develops as a result of insufficient supply of water to the peripheral transpiring parts, as a result of insufficient centrifugal diffusion of water through the leaf. It remains an open question as to where the greatest diffusion gradient is located.

The situation as regards stomatal transpiration is more difficult. The question is also connected with the problem of the relative humidity of the air in intercellular spaces when the stomata are open. Existing views to the effect that air inside the intercellular spaces is practically saturated with water vapour are not, it would appear, definitive. Klemm (1956) assumes, on the basis of model experiments, that the relative air humidity in the intercellular spaces may be considerably lower. The question of the influence of "incipient drying" in the original static conception has come up again with the discovery of suberin layers in the intercellular cell walls. I consider, however, that the controlling influence of water deficit on the intensity of stomatal transpiration is not bound only to the intercellular analogy of the static conception of "incipient drying" of the cuticle, but that it is now possible to conceive this influence from the dynamic point of view in the same way as it can be assumed with regard to cuticular transpiration.

The very highly significant negative correlation obtained in our experiments between the rate of increase of water deficit and pure stomatal transpiration provides further proof of the substantial influence of the dynamics of water deficit on the intensity of stomatal transpiration. To a similar extent as in cuticular transpiration this influence primarily depends on the DPD gradient between the transpiring surface and the inner layers, or cell contents. A proof of the existence and influence of this gradient is provided by the difference that has been found between the magnitude and the significance of the correlation of intensity of stomatal transpiration and the rate of water deficit on the one hand and the rate of increase of water deficit in the leaf on the other hand. If we consider only the static aspect, then according to Renner's analysis the water vapour tension at the surface of the intercellular cell walls could only during the loss of turgor of the cells limiting the intercellular spaces be reduced to an extent corresponding with the osmotic pressure of the cell sap. This reduction would probably be insufficient to bring about any substantial decrease in the transpiration from the intercellular cell walls. But if we consider the dynamic aspect of the process, then the conception of such a mechanism of control of intercellular transpiration as we know it from the explanation of the control mechanism of cuticular transpiration (i.e. primarily as a result of reduction in permeability to water) is quite logical.

The regression coefficients b_y calculated from our experiments and given in Tables 2 and 3 provide evidence as regards the quantitative aspect of the influence of water deficit on the intensity of cuticular and stomatal transpiration. It can be seen from them and from the regression lines in Figures 1 and 2 that under our experimental conditions both phases of transpiration, the cuticular and the stomatal, were affected to practically the same extent

by the existence of water deficit. For every rise in the rate of increase of water deficit by 1 % per hour the intensity of both phases of transpiration was reduced by about 7 %. It is, however, necessary to assume that the dependence of the intensity of transpiration on water deficit is not strictly linear throughout when values for the deficit are high.

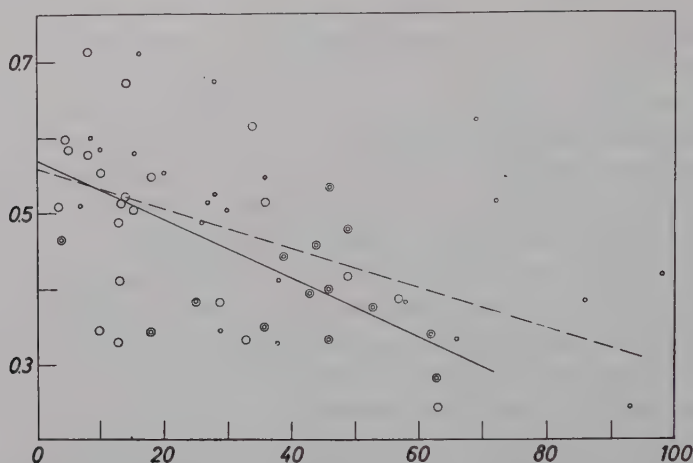


Figure 1. Relation of intensity of stomatal transpiration (ordinate: in $\text{mg. g}^{-1} \cdot \text{min}^{-1}$) to the magnitude (small points), and rate of increase (large points) of initial water deficit (abscissa: in per thousand, resp. in per thousand per hour) in experiment A. Regression lines of relation of stomatal transpiration to the magnitude (—) and the rate of increase (---) of initial water deficit.

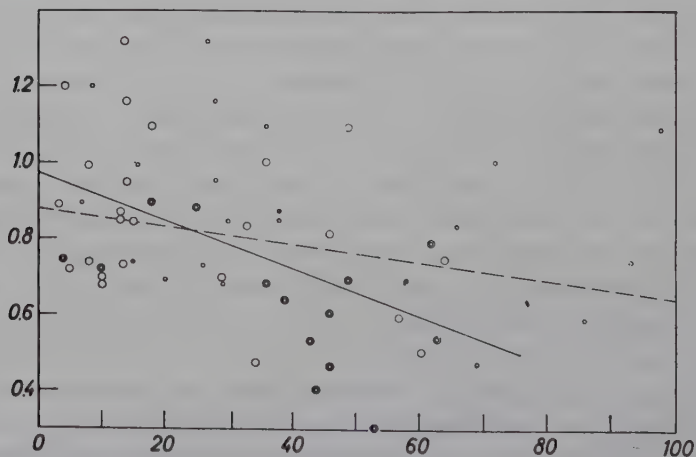


Figure 2. Relation of intensity of cuticular transpiration (ordinate) to the magnitude (small points) and the rate of increase (large points) of the initial water deficit (abscissa) in experiment A. Regression lines of relation of cuticular transpiration to the magnitude (---) and the rate of increase (—) of initial water deficit.

Summary

1. Quantitative analysis of loss curves (Hygen, 1951) for sugar-beet leaves was used for contributing to the problem of the influence of water deficit on the intensity of stomatal and cuticular transpiration.

2. In all experiments a highly significant negative correlation was found between the intensity of cuticular transpiration, calculated from transpiration curves and reduced to the zero initial deficit, and the initial water deficit of the leaf (deficit 0 to 10 %). Still higher, also very significant correlation coefficients were calculated between this converted intensity of cuticular transpiration and the rate of increase of the initial deficit, *i.e.* the magnitude of the initial total deficit divided by the time during which this deficit developed.

3. In the same series of experiments, although the negative correlation coefficient between pure stomatal transpiration reduced to zero water deficit and the initial water deficit of the leaf was not found to be significant, nevertheless there was a highly significant negative correlation coefficient between this stomatal transpiration and the rate of increase of the initial water deficit.

4. The existence of the significant correlation coefficients quoted indicates that both the intensity of cuticular transpiration and also the intensity of the stomatal phase of transpiration *is* indirectly related not only to the magnitude of the water deficit of the transpiring leaf, but also clearly to an even greater degree on the rate of increase of this deficit. This second fact is evidence that, similarly to cuticular transpiration, stomatal transpiration also is primarily affected by the dynamic component of water deficit, *i.e.* by the origin and consequences of the DPD gradient on the centrifugal diffusion path of water to the transpiring outer surfaces. It is clear from this that the water vapour tension at the outer surface of intercellular cell walls does not correspond to the the total water deficit of the leaf.

5. According to the regression coefficients, the intensity of stomatal transpiration was reduced in our experiments for every 1 % of water deficit by about 3 %, cuticular transpiration by 6 %. For every rise in the rate of increase of water deficit of 1 % per hour both cuticular and stomatal transpiration decreased by an average of 7 %.

6. The fact that water deficit influences stomatal transpiration is a further indirect proof of the hypothesis that the relative humidity of the air in the intercellular spaces may be lower than the assumed nearly 100 %.

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Aspartic-C¹⁴ Acid Metabolism in Leaves, Roots, and Stems

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Evidence has accumulated from experiments with bacteria, animal, and plant tissues indicating that aspartic acid, like glutamic acid, is an important link between carbohydrate and protein metabolism. A major portion of the earlier work on aspartic acid metabolism in plants was reviewed by Chibnall (1939); more recent findings have been summarized by Meister (1957). It is thought that aspartic acid participates in a number of metabolic pathways, in addition to being utilized in protein synthesis and entering into the tri-carboxylic acid cycle via transamination. Aspartic acid may be transformed directly into the amide, asparagine (Chibnall, 1939). Frequently it has been stated that β -alanine, a moiety of coenzyme A, is derived from aspartic acid by decarboxylation (Bonner, 1950). Another decarboxylating enzyme in *Clostridium welchii* has been shown to yield α -alanine (Meister *et al.*, 1951). Furthermore evidence has accumulated indicating that threonine is derived from aspartate by way of homoserine (Black and Wright, 1955; Watanabe *et al.*, 1957).

These investigations with C¹⁴-labeled aspartic acid were undertaken to determine the pathways of aspartic-acid metabolism in different plants and which of these pathways are seemingly most active. Furthermore we looked

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for possible side reactions that might shed light on unsuspected pathways of biosynthesis of important metabolites arising from aspartic acid. Aspartate C^{14} was supplied to detached leaves, hypocotyls, and terminal root-tip segments of a number of species. The leaves were administered labeled aspartic acid in either light or in darkness in an atmosphere of air or nitrogen. The carbon label was followed by sampling of the tissue at different intervals. The general pattern of metabolism of aspartic acid seemed to be similar in all of the species investigated. Leaves and roots were strikingly different in the biosynthesis of asparagine and homoserine.

Materials and Methods

Experimental methods and techniques used were similar to those used by Naylor and Tolbert (1956). Detached leaves or leaflets of Sacramento barley, Thatcher wheat, Blood turnip, red beet, Big-Boy tomato, Alaska pea, white lupine, Kudzu (*Pueraria lobata*), 1-cm. terminal root-tip segments of Sacramento barley, Blue Bonnet rice, Forkeddeer oats, maize (OH-51a×B8), white lupine, Alaska pea, Hawkeye soybean, Black Valentine bean, buckwheat, and stems of Alaska pea, Hawkeye soybean, alfalfa, and peppermint plants were used. Bases of blades or the petioles of leaves or leaflets were submerged 1—2 mm. in an aqueous solution of the aspartic- $U-C^{14}$ acid in a small, pointed test tube. Twenty μ l. of solution having 0.77 mg. aspartic acid with a total activity of 1 μ c. provided sufficient label in a 180—200-mg. leaf over the time interval for the subsequent analyses. As soon as the solution was absorbed by the leaf, water was added to the tube. One-cm. root tips from seeds germinated about 1 week were immersed in an aspartic-2,3- C^{14} acid (0.5 μ c.) solution in a 30-mm. porcelain combustion boat. Short segments of coleoptiles, hypocotyls, and stems were treated similarly to leaves.

After the treatment, leaves were killed by quick immersion in boiling 40-per-cent methanol water where they remained for approximately 1 minute. The leaves were further extracted with water by being thoroughly ground with a small mortar and pestle. The insoluble material was removed by centrifugation. The alcohol and water extracts were combined and evaporated at room temperature to 0.1 ml. with a stream of dry, filtered air. Ten to 20 μ l. of this concentrated extract, the equivalent of 20—40 mg. of fresh tissue, made satisfactory chromatograms. Root tips, coleoptile, hypocotyl, and stem segments were killed in 80 per cent ethanol.

Results

Metabolism in Leaves. — The major water- and alcohol-soluble compounds deriving a portion of their carbon skeletons from aspartic acid in relatively short intervals under aerobic or anaerobic conditions were similar for all plants used (Tables 1 and 2). This is illustrated in more detail in Table 1 where data from analyses of four representative plants are recorded. Utilization patterns were remarkably uniform in the leaves of the several species examined.

Table 1. Aspartate C¹⁴ metabolism by excised leaves.

Experimental conditions	Percentage aspartic acid metabolized	Percentage distribution of C ¹⁴ metabolized from aspartic-C ¹⁴ acid											
		Malic	Citric	Succinic	Glutamic	Glutamine	Unidentified under glutamic	γ-Amino butyric	Alanine	Threonine	Asparagine	β-Alanine	Others ¹
Sacramento Barley													
1 hr.-light-air	53.6	74.7	1.9	0.4	3.1	0	11.2	0.8	1.7	1.6	0	0	4.4
3 hrs.-light-air	89.9	73.9	1.9	0.3	4.8	0	11.1	1.1	1.3	1.4	trace	0	4.0
1 hr.-dark-air	17.3	50.6	1.6	1.4	26.7	0	1.6	1.2	3.7	1.0	0	0	1.7
3 hrs.-dark-air	35.9	58.3	5.9	1.7	20.1	trace	5.9	1.4	4.4	0.8	0.4	0	0.3
3 hrs.-light-N ₂	10.1	61.8	1.8	2.4	8.5	3.0	1.8	1.8	13.9	3.0	0	0	0
6 hrs.-light-N ₂	22.7	56.9	1.2	5.0	7.5	1.4	1.2	1.3	14.0	1.2	0.7	0	8.7
3 hrs.-dark-N ₂	4.5	56.8	0	6.8	trace	11.4	0	4.5	4.5	0	0	0	15.9
6 hrs.-dark-N ₂	7.4	54.1	0	5.4	trace	6.8	0	5.4	5.4	0	0	0	23.0
Thatcher Wheat													
1 hr.-light-air	33.1	51.8	1.8	1.5	8.7	14.9	trace	2.6	6.3	1.4	0	0	11.1
3 hrs.-light-air	89.4	57.5	2.0	0.6	6.0	20.2	?	0.4	1.9	1.0	0.2	0	10.0
1 hr.-dark-air	12.1	23.3	2.7	2.1	34.4	6.1	trace	7.0	16.3	trace	0	0	8.0
3 hrs.-dark-air	15.7	41.5	3.7	1.5	20.9	13.2	trace	2.4	10.3	0.7	1.5	0	4.2
3 hrs.-light-N ₂	19.2	44.8	1.4	2.4	11.3	2.8	trace	1.4	27.8	2.6	0	0	5.4
6 hrs.-light-N ₂	23.7	45.2	1.4	2.9	8.8	1.7	0.9	1.7	26.8	2.3	0.9	0	7.4
3 hrs.-dark-N ₂	4.7	45.7	0	14.3	trace	0	9.5	7.6	15.2	0	0	0	7.6
6 hrs.-dark-N ₂	4.4	52.9	0	10.7	trace	0	6.6	9.9	14.0	0	0	0	5.8
Tomato													
1 hr.-light-air	22.0	77.0	9.0	0	9.0	9.0	0	0	trace	0	0	0	0
3 hrs.-light-air	83.0	81.0	2.0	0	2.0	10.0	0	0	0	0	0	0	6
6 hrs.-light-air	83.0	77.0	7.0	0	7.0	6.0	0	0	0	0	0	0	10
1 hr.-light-N ₂	9.0	63.0	0	0	0	trace	0	0	25.0	0	0	0	0
3 hrs.-light-N ₂	19.0	86.0	0	0	0	trace	0	0	14.0	0	0	0	0
6 hrs.-light-N ₂	26.0	90.0	trace	0	trace	trace	0	0	10.0	0	0	0	0
White Lupine													
15 mins.-light-air	16.0	91.8	1.2	—	1.2	trace	0	0	1.2	0	0	0	4.6
30 mins.-light-air	33.5	78.7	3.5	—	3.5	7.1	0	0	3.5	0	0	0	3.7
1 hr.-light-air	61.4	63.6	5.1	trace	3.8	17.2	0	1.3	3.4	0	0	0	5.6
3 hrs.-light-air	81.3	76.6	2.8	1.4	6.6	3.7	0	0.9	0.9	0.9	0	0	6.2
1 hr.-dark-air	25.7	51.8	12.4	0.7	17.9	2.9	0	2.3	3.6	0	trace	0	8.4
3 hrs.-dark-air	24.0	38.2	12.4	trace	34.1	5.0	0	7.0	1.7	0	1.6	0	0
1 hr.-light-N ₂	25.9	48.0	trace	2.0	trace	0	0	0	46.0	0	2.0	0	2.0
3 hrs.-light-N ₂	34.1	46.2	trace	19.2	2.6	0	0	trace	32.1	0	0	0	0
3 hrs.-dark-N ₂	7.2	26.8	0	28.2	0	0	0	trace	31.1	0	0	0	14.0
6 hrs.-dark-N ₂	8.6	32.3	0	17.2	0	0	0	3.2	38.7	0	0	0	18.6

¹ These figures include small amounts of glycine, fructose, serine, and cysteic acid. Where sucrose is formed, it is also included in these figures.

Table 2. *Comparative metabolism of aspartic-C¹⁴ acid by various species in the dark and in air.*

Plant	Organ	Time (hrs.)	Percentage aspartic acid metabolized	Percentage distribution of C ¹⁴ in products from aspartic acid metabolized ²					
				Malic	Glutamic	γ -Amino-butyric	Glutamine	Asparagine	Alanine
Barley	Roots	2	81.3	33.9	14.2	6.6	31.8 ¹	6.9	2.0
	Coleoptile	2	64.8	14.6	23.2	7.5	16.6	15.3	17.3
	Leaves	3	35.9	58.3	20.1	1.4	trace	0.4	4.4
Rice	Roots	2	75.7	36.9	20.9	3.7	26.7	0.7	6.3
Oats	Roots	3	38.6	28.5	14.8	4.6	14.8	24.3	3.4
	Coleoptile	2	42.5	13.2	19.8	3.2	18.7	31.7	4.9
Wheat	Leaves	3	15.7	41.5	20.9	2.4	13.2	1.5	10.3
Corn	Roots	2	42.6	21.9	20.7	10.4	6.8	6.4	4.0
	Coleoptile	2	62.3	43.2	20.0	2.2	2.5	4.3	2.5
Lupine	Roots	1.5	26.8	47.0	14.9	4.5	4.5	4.0	7.9
	Leaves	2	39.2	57.5	15.2	1.6	4.7	1.8	1.6
Peas	Leaf	2.5	66.7	21.1	20.1	5.3	4.7	0.5	8.0
	Fruit	2.5	72.3	22.3	13.9	7.6	9.7	3.1	6.9
	Root	3	79.4	28.4	20.2	1.1	7.3	10.0	12.4
	Stem	2	35.6	39.4	10.6	14.4	3.5	2.3	21.6
Soybean	Roots	2	28.9	29.0	11.6	15.9	8.7	4.3	5.8
	Stems	2	48.0	40.6	3.3	15.2	1.4	trace?	7.6
Alfalfa	Stems	2	43.5	23.6	9.7	23.0	3.0	2.7	24.7
Kudzu vine	Leaves	1	35.5	35.6	26.1	2.4	1.9	0.0	2.4
Buckwheat	Roots	2	90.8	19.7	11.9	trace	58.5 ¹	trace	trace
Mint	Stem	2	44.0	38.6	2.0	12.8	4.5	0.7	5.3

¹ Includes pyrrolidone carboxylic acid derived from glutamine during preparation for chromatography.

² Labeling in other compounds, e.g. citric and succinic acids, represents the difference between 100 % and the sum of percentages listed.

Table 1 shows that added aspartate was used at a faster rate in light than in the dark. Part of this increase might be attributed to faster uptake of the solution by the leaves in the light; however, in both light and darkness all of the radioactive solution was absorbed within an hour. Air also promoted utilization of the aspartate. Malate received more label than any other compound in the shortest aerobic experiment (15 minutes). In this interval, citrate, glutamate, and alanine received some label. There was no indication that oxygen was absolutely necessary as an ultimate hydrogen acceptor in any of the reactions leading to appearance of C¹⁴ label in malate. In contrast, there was a definite relation between the presence of air and the appearance of label in citrate. Without exception, more label from aspartate accumulated in citrate in darkness and in air than under any other condition. In darkness

and in a nitrogen atmosphere, appreciable amounts of C¹⁴ label did not appear in citric acid, this being consistent with the fact that oxygen is necessary for operation of the citric-acid cycle. C¹⁴ label did appear, however, under anaerobic conditions in succinic acid, in glutamine, and in γ -aminobutyric acid. Though the percent of the total aspartic acid that was converted anaerobically into these products was appreciable, the amount was very small since the metabolism of aspartate in the dark and in a nitrogen atmosphere was slow.

Glutamic acid consistently accumulated more C¹⁴ label in the leaves in the dark with air than in the light. No label appeared in the glutamic acid under conditions of darkness and a nitrogen atmosphere. This may be taken as evidence that oxygen is important in the production of glutamic acid from aspartic acid. Darkness as well as a nitrogen atmosphere usually seemed to favor accumulation of label in γ -aminobutyric acid (Naylor and Tolbert, 1956). In general, glutamine was most strongly labeled when the leaves had both light and air; whereas, under the same conditions, asparagine became labeled from aspartic-C¹⁴ acid much more slowly. The label appeared in asparagine more readily in the dark than in the light in air.

Alanine accumulated label from aspartic acid under a nitrogen atmosphere as well as in air. With the reduced rate of aspartate conversion in a nitrogen atmosphere taken into consideration, there was probably no increase in alanine labeling under anaerobic conditions. Some label appeared in threonine in these plants but none was detectable in homoserine.

No evidence of label transfer to β -alanine was obtained in any of the plants tested (Tables 1 and 2). In none of the plants, however, except Kudzu, could unlabeled β -alanine be detected by ninhydrin treatment of the paper chromatograms. But even in Kudzu leaves, where the β -alanine pool was readily detectable, label from aspartic acid was not found after 6 hours.

Metabolism in Roots, Stems, and Coleoptiles in Comparison with Leaves.

— During administration of radioactive aspartate, roots, stems, and coleoptiles were kept in the dark in air. Data from these experiments are recorded in Table 2. All the tissues tested utilized aspartic acid; buckwheat, barley, and pea roots transformed it more rapidly than any other tissues; lupine and soybean roots were slowest. Most of the label from aspartate quickly found its way into Krebs cycle intermediates and their derivatives and particularly into glutamic acid and its family of compounds.

Buckwheat, barley, and rice roots transformed a considerable amount of the aspartate into glutamine. Of all the tissues tested, oat roots and coleoptiles converted the most C¹⁴ of aspartate into asparagine. Barley coleoptiles, and

Table 3. *The formation of homoserine from aspartic-C¹⁴ acid in excised parts of Alaska pea plants in air and in darkness.*

Part	Length of test (hr.)	% of total metabolized aspartic acid converted to homoserine
Leaf	2.5	0.6
Young fruit	2.5	3.5
Stem	2.5	None detected
Roots.....	3	15.5

pea, barley, and corn roots labeled asparagine from aspartic acid in lesser amounts. Lupine, which is generally considered to be a prime producer of asparagine, had as much label in glutamine as in asparagine. Qualitative ninhydrin spray tests indicated that the reservoir of asparagine in lupine was larger than that of glutamine. Therefore, it is probable that the specific activity of glutamine was higher than that of asparagine even though aspartic-C¹⁴ acid was the labeling material. In peas, barley, and lupine the data also indicate clearly that asparagine obtains label from aspartic acid more readily in roots than in leaves (Table 2).

Alanine became highly labeled in barley coleoptiles, pea and alfalfa stems, and wheat leaves. More radioactive carbon from aspartic acid was accumulated in alanine of the stems than in any other tissue. In stems more label accumulated in γ -aminobutyric acid than in leaves or roots; perhaps this indicates an active glutamic decarboxylase.

A radioactive amino acid, present in pea roots after aspartic acid-2,3-C¹⁴ was administered, cochromatographed with synthetic homoserine. Homoserine has been reported to occur in sizeable concentrations in peas and other plants (Virtanen and Miettinen, 1953; Miettinen, 1955; Berg *et al.*, 1954). We confirmed this, and in addition found that pea leaves and young fruits were capable of synthesizing homoserine from aspartate, but stems were not. A comparison of the activity of the different parts of the pea plant in carrying out this synthesis is illustrated in Table 3. The roots were far more active in synthesis than the fruits or leaves. In none of the organs of the pea plant were detectable amounts of label found in threonine, though there were pools of threonine of sufficient size to be detectable with the ninhydrin spray.

Discussion

In the short-term experiments more label accumulated in malic acid from labeled aspartic acid than in any other substance. Although numerous plant species were used, the preponderance of the label from aspartate always

accumulated in malic acid but never in succinic, citric, or any other of the organic acids known to accumulate in plants. Within 30 minutes to 1 hour, equilibrium conditions seemed to be established between the added aspartate and those substances deriving label from it; this was indicated by the relatively constant percentage distribution of C¹⁴ among the products. Since so much of the label from aspartate appeared in organic acids associated with the Krebs cycle and in substances belonging to the glutamic acid family of compounds, there is little doubt that movement of label from aspartate into the citric-acid cycle is favored over alternative pathways.

The rapid appearance of label in malate can be interpreted in at least three ways. Possibly there is an aspartase present that can convert aspartic acid to fumaric acid; then subsequent hydration leads to the formation of malic acid. Aspartase has been demonstrated in crushed, nodulated roots of peas, in leaves of red clover (Virtanen and Tarnanen, 1932); it has been purified from *Propionibacterium peterssonii* (Ellfolk, 1955) and *Escherichia coli* (Ichihara *et al.*, 1955). Since 1932 aspartase has been assumed but not demonstrated to be widely distributed in plants. Another way in which carbon label from aspartate could reach malate is through oxalacetic acid, which is readily reduced to malic acid by reduced coenzyme I. This reaction could be accelerated in the light from the photosynthetic production of the reduced coenzyme. Oxalacetic acid could be formed from aspartic acid either by transamination or oxidative deamination. On transamination of aspartic acid with α -ketoglutarate, labeled oxalacetic and unlabeled glutamic acid would be formed. The presence of an α -ketoglutaric-aspartic acid transaminase in higher plant tissues is well documented (see, Wilson *et al.*, 1954). Oxidative deamination presumably would be dependent on a flavoprotein with oxygen as the ultimate electron acceptor or aspartic acid dehydrogenase, with pyridine nucleotide being the hydrogen acceptor. There is evidence that enzyme systems facilitating these reactions exist in seed plants (Virtanen and Erkama, 1938) and the fungi (Bender and Krebs, 1950; Burton, 1951). Any one of the three suggested routes to malate may be open, but clear-cut evidence indicating any one of them is not provided by the experiments described here.

There was no rapid conversion of aspartate to carbon dioxide in the light; this is indicated by very small amounts of C¹⁴ in photosynthetic products. As with glutamic acid (Naylor and Tolbert, 1956), the over-all rate of metabolism of aspartic acid was several-fold faster in the light than in the dark. It is possible that light might affect respiration and, indirectly, aspartic-acid metabolism through side effects of photosynthesis. In an examination of our data for such a possibility, the implications of the lower percentage of the aspartic acid metabolized in the dark must be considered in conjunction with

the higher percentage distribution of the C^{14} into the immediate products of the citric-acid cycle. As with glutamic acid, the data on aspartic metabolism suggest that in the light the respiration rate was not significantly decreased.

Asparagine has long been assumed to be biosynthesized chiefly from aspartic acid. Mothes (1933), for example, reported this in excised leaves. Wheat germ and white lupine seedling extracts were reported (Webster and Varner, 1955) to carry out asparagine synthesis in the presence of aspartate, ammonium, and magnesium ions. In our experiments, the carbon label derived from aspartic acid appeared rather slowly in the asparagine of excised leaves, only small amounts being present after 6 hours. These results were not unlike those of Nelson *et al.* (1953), which showed that after 6 days of photosynthesis in a $C^{14}O_2$ atmosphere, comparatively little radioactivity was present in asparagine of *Lupinus angustifolius*. Although there can be no doubt that excised leaves are capable of synthesizing asparagine, it is equally clear that roots have a much greater capacity to produce this amide and possibly supply much of that which accumulates in the leaves. Although the legumes are ordinarily thought to be prime producers of asparagine, oat roots and barley and oat coleoptiles transferred proportionally a higher percentage of the label from aspartate to asparagine than any of the legumes. In these experiments in which it was expected that asparagine synthesis would be favored, more label from aspartic acid appeared in glutamine than in asparagine. Only little radiocarbon from aspartate was found in asparagine in the leaves; the labeling in glutamine was substantial. Whereas glutamine labeling in the leaves was accelerated in the light and air, asparagine labeling was negligible and increased in the dark. Thus glutamine seemed to be a product more characteristic of light conditions and asparagine more of dark conditions.

Label from aspartic acid appeared in the threonine pool in the shortest time interval. Presumably the pathway of threonine synthesis is the same in higher plants as that described by Black and Wright in yeast and in *E. coli* (1955). They found that aspartic acid was converted successively into β -aspartylphosphate, β -aspartic semialdehyde, homoserine, and eventually into threonine. Though threonine was detected in most of the plants tested, homoserine was not detectable by ninhydrin or radioactivity except in the pea plant. In the roots of peas, homoserine was a major product from aspartic acid; threonine remained unlabeled. Although a large homoserine pool was present in the pea stem, labeled aspartate was not transformed to homoserine by the pea stem segments. Very likely the homoserine in the stem was synthesized in another organ, possibly the roots.

We have previously reported (Naylor and Tolbert, 1956) a marked change in metabolism of glutamic acid when plant tissue is placed under anaerobic

conditions. γ -Aminobutyric acid, which possibly arose through the action of glutamic decarboxylase, accumulated in quantity. Likewise decarboxylation of aspartic acid could lead to the formation of β -alanine. Since β -alanine is a part of pantothenic acid, which is itself a precursor of coenzyme A, special significance is attached to the origin of β -alanine.

Several years ago, it was suggested that β -alanine secreted from pea root nodules might be derived from aspartate through decarboxylation (Virtanen and Laine, 1937; and Virtanen, Rintala, and Laine, 1938). β -Alanine was reported in a number of higher plant tissues (see Hulme and Arthington, 1952; Miettinen, 1955; Champigny and Lioret, 1955; Mansford and Raper, 1956). We have found it in leaves of the Kudzu vine; we obtained no evidence, however, that β -alanine derives label from aspartic acid *in vivo* within 6 hours.

Our inability to demonstrate the origin of β -alanine from aspartic acid may be accounted for in several ways. It may be that the pool size is so small and the rate of utilization so great in young growing tissues that our procedures could not detect it. This possibility will not account for the fact that the β -alanine pool in Kudzu leaves was not labeled by aspartic acid. Possibly β -alanine is not derived from aspartic acid in higher plants; also it may be that the β -alanine moiety of coenzyme A is not derived from the free amino acid. Recent work (Stadtman, 1956) indicates that β -alanine may be formed from propionyl coenzyme A. In our experiments, since the label from aspartic acid was introduced into all the components of the citric acid cycle, β -alanine was not being synthesized readily from any of these compounds.

Summary

1. Aspartic-C¹⁴ acid was readily metabolized in excised organs of a wide variety of plant species. In leaves, both light and aerobic conditions accelerated the disappearance of label from the supplied aspartate.

2. Label from aspartate-C¹⁴ was quickly converted into malate under both aerobic and anaerobic conditions.

3. Aerobic conditions favored the labeling of citric acid, glutamic acid, glutamine, asparagine, and threonine. Under anaerobic conditions, a higher percentage of the aspartate metabolized was found in succinic acid.

4. Alanine became labeled readily under aerobic and anaerobic conditions.

5. Glutamine was labeled readily in the leaves and roots. Less label from aspartate appeared in asparagine than in glutamine. Glutamine labeling from aspartate was greater in the leaves in light; asparagine labeling was much less. In darkness formation of asparagine-C¹⁴ increased but never to the

extent as did glutamine-C¹⁴. Roots and coleoptiles incorporate more label from aspartate into asparagine than do leaves. Oat roots and coleoptiles incorporated more label into asparagine than any of the legumes tested.

6. Homoserine and threonine derived label from aspartate-C¹⁴. Alaska pea roots were especially capable of accumulating label in homoserine but not in threonine. In contrast, all other plants tested accumulated labeled threonine.

7. No evidence was obtained to support the hypothesis that β -alanine is formed from aspartate.

The authors wish to acknowledge the able participation of Mrs. Lola Carter and Miss Pat Kerr in portions of this research.

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Photostade et spectrostade

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Photoperiodisme et photostade

Une des questions fondamentales touchant à la physiologie du développement est évidemment celle du déterminisme de la floraison.

Un grand nombre d'auteurs l'ont abordée plus particulièrement sous l'angle du thermophotopériodisme.

L'état actuel des travaux concernant l'influence du photopériodisme révèle l'existence de points encore insuffisamment éclaircis et qui font l'objet de nombreuses discussions.

Ces dernières années les problèmes du photopériodisme ont été souvent étudiés sous l'angle des interactions entre la qualité du spectre lumineux et la durée quotidienne de son action.

En effet, de nombreux auteurs pensent encore que la durée du jour et la qualité du spectre lumineux sont deux aspects d'un même phénomène: le photopériodisme.

Or sur l'étendue de la période d'action du photopériodisme, deux tendances s'affirment. L'une soutient que cette période s'étend de la fin de la vernalisation à la floraison (Chouard, 1, Lang, 7, Liverman, 8, Wellensiek, 20, Went, 21—22, etc.). L'autre affirme que la durée des processus photopériodiques — en tant que facteurs déterminants de la floraison — ne s'étend que pendant un stade du développement: le photostade. Ce dernier serait suivi d'autres stades au cours desquels la durée du jour serait un facteur secondaire (Fedorov, 2, Kouperman, 3—6, Mathon, 10—13, Sironval, 15, Stroun, 16—19, etc.).

Durée du photostade

Au cours de nos travaux précédents (16—19), nous avons cherché, entre autre, à délimiter la durée du photostade chez les *céréales de jour long*.

Considérer uniquement l'apparition de la fleur ou de l'épi comme preuve de l'effet positif des processus photopériodiques ne permet pas de distinguer les limites de ces phénomènes par rapport à ceux de l'embryogénèse florale. En effet ainsi on ne juge de l'efficacité du facteur photopériodique que bien après qu'il a cessé d'exercer son rôle régulateur.

Aussi nous avons établi une échelle des étapes de l'organogénèse des primordia floraux, échelle que nous avons dernièrement adaptée à celle de Kouperman qui a l'avantage de correspondre nettement à différentes étapes de l'organogénèse des cônes de croissance des céréales (Figures 1—4).

A l'aide de ce critère morphologique nous avons cherché à établir la correspondance entre la durée des processus photostadiaux et certaines étapes de l'embryogénèse florale.

Une des techniques utilisées pour délimiter la durée du photostade consiste à mettre temporairement en jour court, à différentes époques de leur développement, des plantes de jour long se trouvant sous un éclairage quotidien de longue durée.

Dans cette technique il faut considérer:

- a) un stade déterminé du développement qui est celui de la réceptivité des plantes aux phénomènes photopériodiques.
- b) de courtes périodes d'épreuve imposées aux plantes expérimentées successivement au cours du développement.

Si la période d'épreuve se situe antérieurement ou postérieurement à la phase de sensibilité photostadiale, les sujets ne réagiront pas à ce test.

Si la période d'épreuve entame à son début ou à sa fin la phase de sensibilité, les sujets présenteront un retard dans le développement.

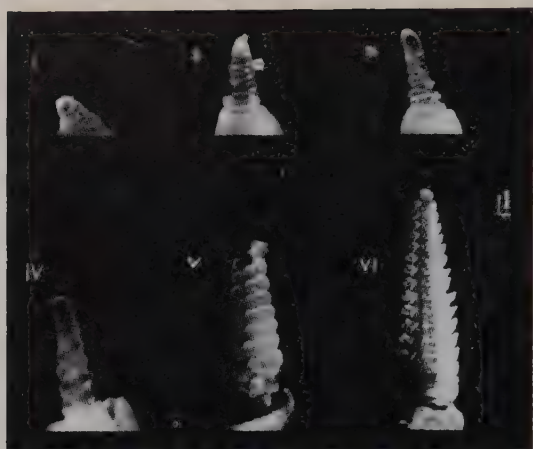
Si la période d'épreuve est comprise entièrement dans la phase de sensibilité, le retard dans la différenciation du primordium floral sera maximum.

L'expérience suivante nous donne un exemple de ce travail.

Le 10.V. 54, nous avons semé, en pots, du Blé de printemps «Roussia» (*T. durum*) qui est placé en jour naturel (du lever au coucher du soleil). Tous les 5 jours, une partie de ces plantes est successivement mise en jour de 8 heures (8.00—16.00) pendant 10 jours, puis ramenée en jour naturel. Les plantes témoins restent en permanence en jour naturel.

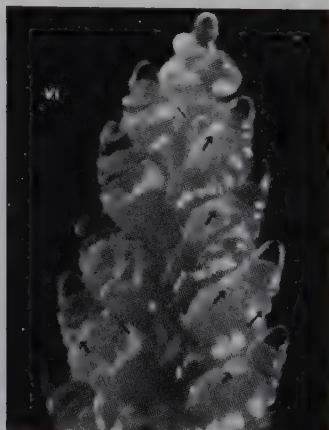
Les résultats qui figurent dans le Tableau 1 montrent:

- a) *des périodes de jour court se situant antérieurement ou postérieurement*



Figures 1 et 2. *Etapes de l'organogénèse du primordium floral de Blé*. Les primordia sont vus de profil — 90° — aux articulations de l'axe du rachis.

- I. — Première différenciation du cône de croissance (émergence du premier bourrelet).
- II. — Segmentation du cône de croissance.
- III. — Courbure des ébauches d'épillets.
- IV. — Apparition des bosses d'épillets (segmentations secondaires au niveau des ébauches d'épillets).
- V. — Gonadogénèse (organogénèse du pistile et des étamines).
- VI. — Gamétogénèse (différenciation cytologique aboutissant à la phase gamétophyte)
- VII a. — Début de l'élongation du rachis et croissance des bractées florales.
- VII b. — Suite de l'élongation du rachis et croissance des bractées florales.
- VII c. — Fin de l'élongation, état avant l'épiaison.
- VIII. — Epiaison.



Figures 3 et 4. *Distinction des étapes V et VI*. On distingue facilement la fin de l'étape V (photo III) et donc le début de l'étape VI (photo IV) à l'apparition des étamines (\rightarrow).
Photos Grandchamp.

Tableau 1. *Etapes de l'organogénèse du blé «Roussia» soumis temporairement en jour de 8 heures à différentes époques de son développement (semis le 10.V.54). n=jour naturel c=jour court de 8 heures.*

Séries	Etape de l'organogénèse atteintes le																		
	Mai					Juin							Juillet						
	10	15	20	25	30	4	9	14	19	24	29	7	8	11	14	15	17	19	21
Témoin	n	n	n	n	n	n	n	n	n	n	n	n							
	0	0	0	0	0	II	III	IV	VI	VII a	VII b	n							
A	c	c	c	n	n	n	n	n	n	n	n	n							
	0		0									n							
B	n	c	c	c	n	n	n	n	n	n	n	n	n						
	0	0		0									VIII						
C	n	n	c	c	c	n	n	n	n	n	n	n	n	n	n				
	0	0	0		0										VIII				
D	n	n	n	c	c	n	n	n	n	n	n	n	n	n	n	n	n	n	n
	0	0	0	0		I													VIII
E	n	n	n	n	c	c	c	n	n	n	n	n	n	n	n	n	n	n	n
	0	0	0	0	0		I												VIII
F	n	n	n	n	n	c	c	c	n	n	n	n	n	n	n	n	n	n	n
	0	0	0	0	0	II		III											VIII
G	n	n	n	n	n	n	c	c	c	n	n	n	n	n	n	n	n	n	n
	0	0	0	0	0	II	III		V										VIII
H	n	n	n	n	n	n	n	c	c	c	n	n	n	n	n				
	0	0	0	0	0	II	III	IV		VI					VIII				
I	n	n	n	n	n	n	n	n	c	c	c								
	0	0	0	0	0	II	III	IV	VI			VII b	VIII						
J	n	n	n	n	n	n	n	n	n	c	c	c							
	0	0	0	0	0	II	III	IV	VI	VII a		VIII							

au photostade. Les plantes réagissent comme le témoin en jour naturel (Séries A, I, J).

- b) *des périodes de jour court entamant le photostade à son début et à sa fin.* Par rapport au témoin, la Série B retarde d'un jour à l'étape VIII, ce qui tend à démontrer que le photostade commence juste à la fin de la période de jour court de cette série (vers le 25.V.54). La série H dont la différenciation des primordia floraux est retardée entame le photostade seulement dans les premiers cinq jours de sa période en 8 heures d'éclairement (environ jusqu'au 19.VI. 54, date à laquelle débute la période en jour court de la série I dont le développement est similaire à celui du témoin).
- c) *des périodes de jour court comprises entièrement dans la phase de sensibilité au photostade.* Les périodes respectives de jour court des séries D, E, F, G se situant après le 25.V.54 et avant le 19.VI.54.

Donc sur un cycle de développement de 58 jours (semis-épiaison), la période au cours de laquelle ces plantes semblent être sensibles aux processus photostadiaux ne dure qu'environ 25 jours (25.V.54—19.VI.54).

Ainsi, bien avant l'épiaison, le photopériodisme ne semble plus avoir aucune influence. Au moment de la gamétogénèse (étape VI), les plantes sont déjà insensibles à la durée d'éclairement.

Le fait que les séries comprises entièrement dans la phase de sensibilité au photopériodisme retardent plus par rapport au témoin quand leur période de jour court se situe au début du photostade (séries D, E) que quand elle est placée à la fin (séries F, G), nous permettait de conclure que la sensibilité à la durée de l'éclairement quotidien va en diminuant au cours du photostade.

*

Kouperman, à la lumière de ses travaux, émit l'opinion que le photostade est encore beaucoup plus court que nous ne le suggérons, notre technique ne permettant pas de faire la distinction entre les processus dépendant de la durée du jour et ceux influencés par la qualité du spectre lumineux. Ceci d'ailleurs expliquait, d'après elle, la différence de sensibilité à la durée de l'éclairement quotidien que nous avions remarquée entre le début et la fin de ce que nous pensions être le photostade.

Pour éclaircir cette question — qui est d'une grande actualité au moment où de nombreux chercheurs expérimentent l'influence des radiations de diverses longueurs d'ondes — nous avons repris nos recherches en utilisant les techniques écologiques proposées par Kouperman.

Notre hypothèse de travail admet donc l'existence d'un stade, succédant au photostade, où le facteur déterminant serait la qualité du spectre lumineux. Nous proposons de désigner cette période par le terme «*Spectrostade*».

Photostade et spectrostade

Une difficulté se présente lorsque l'on veut délimiter les phénomènes dépendant, d'une part, de la durée d'éclairement et, d'autre part, de la qualité du spectre lumineux. En effet, tout éclaircissement implique forcément un effet de durée et de qualité lumineuses. Or pour délimiter le photostade et le spectrostade, il est absolument nécessaire de se trouver dans des conditions telles que les caractères de chacun — en tant que facteurs déterminants — puissent être mis en évidence.

Les expériences suivantes répondent, semble-t-il, à ces exigences.

Des plantes de céréales de jour long (Blé, Orge¹) sont mises en jour court (10 heures) à différents moments de la journée (entre 4.00 et 21.00). Ainsi certaines plantes reçoivent la lumière de la première partie de la journée, d'autres du milieu du jour et d'autres de la fin de la journée. Or l'on sait que dans la première partie de la matinée prédominent les radiations à forte longueur d'onde du spectre solaire (dans le rouge et l'infra rouge), au milieu de la journée la lumière est riche en radiations à courte longueur d'onde (dans le bleu) et tout en fin de soirée de nouveau prévalent les radiations à forte longueur d'onde.

Il aurait été préférable de soumettre les plantes seulement à 8 heures d'éclairement afin de rétrécir le champ d'action du spectre solaire; malheureusement des difficultés techniques ne nous ont pas permis d'obtenir moins de 10 heures d'éclairement.

Pour raccourcir le jour chez les différentes séries, on a recouvert les plantes — en dehors des heures d'éclairement — de cages de toile noire.

Pour obtenir les mêmes conditions de température et d'humidité chez les témoins, on a mis sur ces derniers des cages de papier cellophane incolore transparent.

Expériences sur les Blés

1re expérience

Les Blés tendres de printemps *Lichti I* (*T. vulgare*) sont semés le 16.V.57:

en jour naturel: *Témoin* (de 4.00 à 21.00)

en jour de 10 h. $\left\{ \begin{array}{l} \text{Série A (de 4.00 à 14.00)} \\ \text{Série B (de 4.00 à 9.00 et de 16.00 à 21.00)} \\ \text{Série C (de 8.00 à 18.00)} \\ \text{Série D (de 11.00 à 21.00)} \end{array} \right.$

(voir Figure 5)

Les résultats exposés dans la Figure 6 montrent que:

Le développement de toutes les plantes bénéficiant de 10 h. de jour présente un retard par rapport au témoin en jour naturel.

Jusqu'au moment où les sujets en 10 h. atteignent l'étape III de l'organogénèse, ce retard est le même pour tous.

A partir de l'étape III de l'organogénèse les divers régimes de 10 h. produisent des effets différents. Les plantes des *Séries A* et *B* se développent plus rapidement que celles des *Séries D* et *C*. Lors du dernier contrôle, le 23.VII.57, les plantes *A* ont épié, chez celles *B* l'élongation des primordia floraux est terminé, les plantes *D* ont les leurs qui s'allongent encore et celles *C* sont à l'étape de la gamétogénèse. Remarquons que chez les sujets *A* l'étape VI avait été atteinte environ un mois plus tôt (21.VI.57).

¹ Les semences proviennent soit de la Maison «Vilmorin» soit des «Stations Fédérales d'Essais Agricoles».

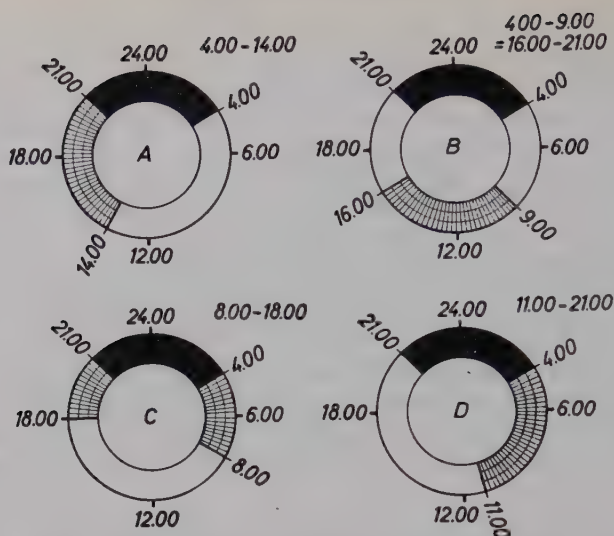


Figure 5. Epoque de la journée pendant lesquelles les plantes reçoivent la lumière naturelle.

Noir: la nuit.

Blanc: époques de la journée pendant lesquelles les plantes reçoivent la lumière naturelle.

Cadrillé: époques de la journée pendant lesquelles les plantes sont recouvertes de cages noires.

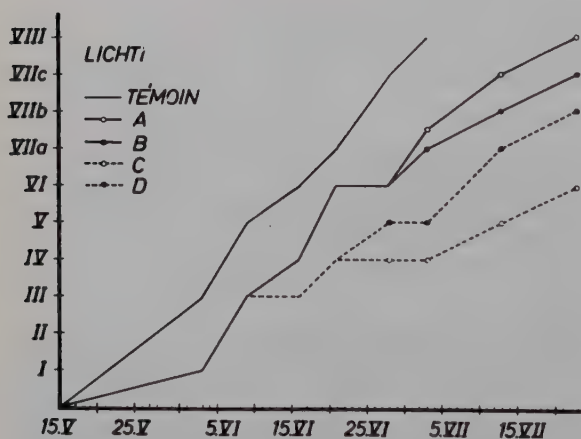


Figure 6. Courbes de la différenciation des cônes de croissance du Blé «Lichti I» soumis à divers régimes de durée et de qualité d'éclairage. Sur l'axe des abscisses les dates de passage des étapes de l'organogénèse.

Témoin

Série A (4.00—14.00)

Série B (4.00—9.00 et 16.00—21.00)

Série C (8.00—18.00)

Série D ((11.00—21.00)

Il résulte de ces constatations que jusqu'à l'étape III de l'organogénèse seule la durée du jour entre en ligne de compte en tant que facteur déterminant. En effet, quelle que soit la qualité du spectre solaire, toutes les plantes de 10 h. ont un développement uniforme.

A partir de l'étape IV (apparition des bosses d'épillets), les plantes sont sensibles à un nouveau facteur. Le comportement des Blés des séries de 10 h. devient différent selon la période du jour à laquelle ils sont exposés.

On voit que la lumière provenant de la partie rouge du spectre est d'une grande importance dès la différenciation des épillets (étape IV). Les plantes ayant reçu la lumière de la première partie de la matinée (*Séries A et B*) se développent plus rapidement que celles du milieu du jour (*Série C*) et de l'après-midi (*Série D*). Ces dernières, d'ailleurs, qui reçoivent pendant une courte période en fin de soirée une quantité prédominante de radiations à forte longueur d'onde se différencient plus rapidement que celles du milieu du jour où seules dominent les radiations d'ondes courtes.

2^{me} expérience

Le Blé alternatif «*Hybride de Bersée*» (T. vulgare) est semé le 16.V.57 (après que les graines aient été vernalisées artificiellement pendant un mois à 2°—3° C. au frigidaire):

en jour naturel: *Témoin* (de 4.00 à 21.00)

en jour de 10 h. $\left\{ \begin{array}{l} \text{Série A (de 4.00 à 14.00)} \\ \text{Série B (de 4.00 à 9.00 et de 16.00 à 21.00)} \\ \text{Série C (de 8.00 à 18.00)} \\ \text{Série D (de 11.00 à 21.00)} \end{array} \right.$

(voir Figure 5)

Les résultats exposés dans la Figure VII montrent que:

Jusqu'à l'étape III les sujets des diverses variantes de 10 h. retardent uniformément par rapport à ceux en jour naturel, exception faite pour les plantes de la *Série B*. Mais n'ayant pas fait d'examen des cônes de croissance entre le 21.V.57 et le 2.VII.57 il est très possible que ces plantes aient atteint l'étape

Figure 7. Courbes de la différenciation des cônes de croissance du Blé «Hybride de Bersée» soumis à divers régimes de durée et de qualité d'éclairement. Sur l'axe des abscisses les dates de passage des étapes de l'organogénèse, sur l'axe des ordonnées les étapes de l'organogénèse.

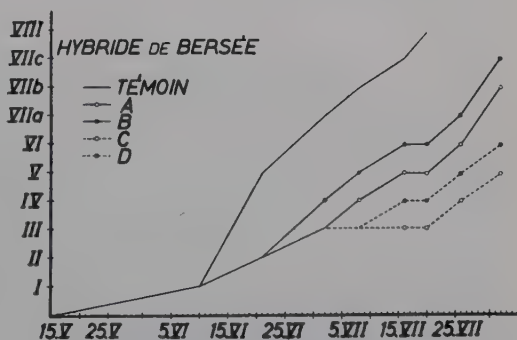
Témoin

Série A (4.00—14.00)

Série B (4.00—9.00 et 16.00—21.00)

Série C (8.00—18.00)

Série D (11.00—21.00)



III en même temps que celles des trois autres séries de jour court, puis aient passé plus rapidement à l'étape suivante.

A partir de l'étape III l'évolution des cônes de croissance chez les sujets de 10 h. varient selon leur période d'exposition au cours de la journée. Finalement lors du dernier contrôle (le 2.VII.57) chez les Blés des *Séries B* et *A* l'élongation du rachis et des bractées a lieu (étapes VII a, VII b), les plantes *D* sont à l'étape de la gamétogénèse (étape VI) et celles *C* à l'étape de la gonadogénèse (V). Remarquons que la *Série B* avait atteint l'étape V environ trois semaines plus tôt.

Il résulte de ces constatations que, de nouveau, jusqu'environ vers l'étape III de l'organogénèse, seule la durée du jour entre en ligne de compte. Par contre, après cette étape, les plantes sont sensibles à la qualité du spectre solaire.

Dès la différenciation des épillets les primordia floraux des plantes exposées à un éclairage riche en lumière rouge (*Séries B* et *A*) se différencient plus vite que ceux des sujets éclairés par une lumière où domine le bleu (*Séries D* et *C*).

3^{me} expérience

Cette expérience diffère un peu des deux précédentes.

Le Blé alternatif *Hybride de Bersée* (*T. vulgare*) est semé (sans vernalisation préalable des graines à basse température, les Blés alternatifs ayant un thermostade ne nécessitant pas obligatoirement de basses températures) le 16.V.57 en jour naturel.

Le 28.VI.57, les plantes qui jusqu'alors s'étaient développées en jour naturel sont mises:

en jour de 10 h. $\left\{ \begin{array}{l} \text{Série A (de 4.00 à 14.00)} \\ \text{Série B (de 4.00 à 9.00 et de 16.00 à 21.00)} \\ \text{Série C (de 8.00 à 18.00)} \end{array} \right.$
(voir Figure 5)

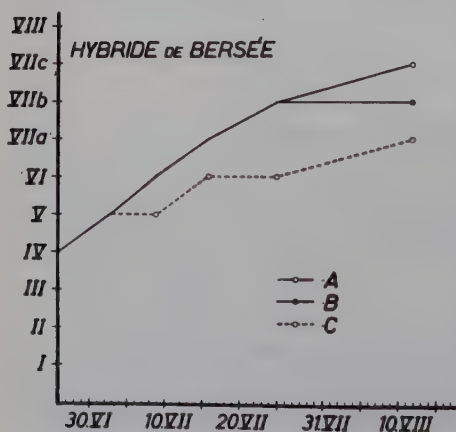


Figure 8. Courbes de la différenciation des cônes de croissance du Blé «Hybride de Bersée» soumis, dès l'apparition des bosses d'épillets à divers régimes de durée et de qualité d'éclairage. Sur l'axe des abscisses les dates de passage des étapes de l'organogénèse, sur l'axe des ordonnées les étapes de l'organogénèse.

Série A (4.00—14.00)

Série B (4.00—9.00 et 16.00—21.00)

Série C (8.00—18.00).

A cette époque les plantes ont atteint l'étape IV de l'organogénèse.

Les résultats exposés dans la Figure 8 montrent que: Les plantes mises tardivement (étape IV) en 10 h. à diverses périodes de la journée n'ont pas un développement similaire. Chez celles de la première partie du matin (*Séries A et B*), les cônes de croissance se différencient plus rapidement que chez celles du milieu du jour (*Série C*).

Ceci montre que la sensibilité à la qualité de la lumière n'est pas un post effet résultant de la mise en jour court de plantes de jour long. Cette réactivité qui s'affirme au moment de l'apparition des bosses d'épillets (étape IV) est spécifique à ce stade du développement.

Expériences sur les Orges

1re expérience

L'Orge de printemps *Isaria* (*H. distichum*) est semée le 16.V.57:

en jour naturel: *Témoin* (de 4.00 à 21.00)

en jour de 10 h. $\left\{ \begin{array}{l} \text{Série A (de 4.00 à 14.00)} \\ \text{Série B (de 4.00 à 9.00 et de 16.00 à 21.00)} \\ \text{Série C (de 8.00 à 18.00)} \\ \text{Série D (de 11.00 à 21.00)} \end{array} \right.$

(voir Figure 5)

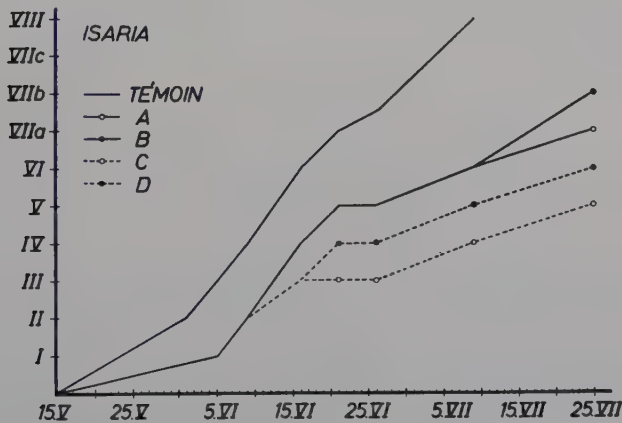


Figure 9. Courbes de la différenciation des cônes de croissance de l'Orge «*Isaria*» soumise à divers régimes de durée et de qualité d'éclairement. Sur l'axe des abscisses les dates de passage des étapes de l'organogénèse, sur l'axe des ordonnées les étapes de l'organogénèse.

Témoin

Série A (4.00—14.00)

Série B (4.00—9.00 et 16.00—21.00)

Série C (8.00—18.00)

Série D (11.00—21.00)

Les résultats exposés dans la Figure 9 montrent que:

Jusqu'à l'étape II de l'organogénèse des cônes de croissance les Orges des variantes de 10 h. retardent également dans leur développement par rapport à celles en jour naturel.

La différenciation des primordia floraux est, par la suite, différente selon les séries de 10 h. (notons qu'une semaine ayant passé entre les contrôles effectués au moment où les plantes étaient à l'étape II et III, il est possible que l'étape III ait été atteinte à peu près en même temps pour toutes les plantes en jour court, puis que le développement se soit brusquement accéléré pour les sujets A recevant la lumière de la première partie du matin).

Lors du dernier contrôle effectué le 25.VII.57, les sujets B sont à l'étape VIIb, ceux A atteignent l'étape VIIa, ceux D sont à l'étape VI et ceux C à l'étape V. Remarquons qu'environ un mois plus tôt les plantes de la première partie du matin avaient effectué leur gonadogénèse.

De nouveau nous voyons que la différenciation des épillets est favorisée par les radiations à forte longueur d'onde du spectre solaire qui prédomine dans la première partie du matin.

2^{me} expérience

L'Orge d'automne *Bordia* (*H. vulgare*) est semée (après que les graines aient été vernalisées artificiellement pendant un mois à 2°—3° C. au frigidaire) le 16.V.57:

en jour naturel: *Témoin* (de 4.00 à 21.00)

en jour de 10 h. $\left\{ \begin{array}{l} \text{Série A (de 4.00 à 14.00)} \\ \text{Série B (de 4.00 à 9.00 et de 16.00 à 21.00)} \\ \text{Série C (de 8.00 à 18.00)} \\ \text{Série D (de 11.00 à 21.00)} \end{array} \right.$

(voir Figure 5)

Les résultats exposés dans la Figure 10 montrent que:

Jusqu'à l'étape III de l'organogénèse toutes les plantes de 10 h. retardent uniformément par rapport aux sujets en jour naturel.

Au moment de l'apparition des bosses d'épillets on note une nette divergence dans la différenciation des cônes de croissance chez les plantes des diverses séries de 10 h. Lors du dernier contrôle, le 25.VII.57, les sujets A et B effectuent la gamétogénèse, les plantes D la gonadogénèse et chez celles C les bosses d'épillets apparaissent.

Ainsi dans cette expérience faite avec une variété d'Orge d'automne nous voyons les mêmes phénomènes que dans les cas de l'Orge de printemps et des Blés: la durée du jour est déterminante jusqu'à l'étape III, puis ensuite

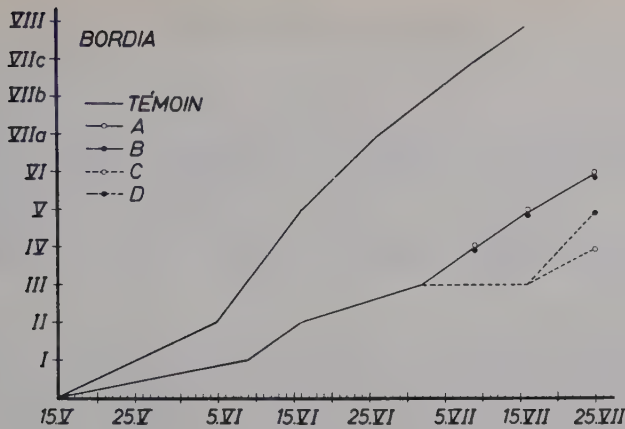


Figure 10. Courbes de la différenciation des cônes de croissance de l'Orge «Bordia» soumise à divers régimes de durée et de qualité d'éclairement. Sur l'axe des abscisses les dates de passage des étapes de l'organogénèse, sur l'axe des ordonnées les étapes de l'organogénèse.

Témoin

Série A (4.00—14.00)

Série B (4.00—9.00 et 16.00—21.00)

Série C (8.00—18.00)

Série D (11.00—21.00).

c'est la qualité du spectre lumineux qui détermine le comportement des plantes de journée longue. Une prédominance de lumière rouge favorise alors leur développement.

En conclusion de ces diverses expériences (nos travaux sur le Seigle ont mis en évidence les mêmes phénomènes que ceux observés sur le Blé et l'Orge), nous voyons que la durée de l'éclairement est le facteur dominant jusqu'à l'apparition des bosses d'épillets. C'est le *Photostade*.

A partir de la différenciation des épillets le facteur dont dépend le développement des plantes est celui de la composition du spectre lumineux. C'est le *Spectrostade*.

Chez les plantes de jour long les processus spectrostadias sont favorisés par une lumière riche en radiations de grande longueur d'onde.

On comprend maintenant pourquoi dans nos expériences précédentes nous avons englobé le spectrostade dans le photostade. En effet en général le jour court que nous avons établi s'étendait de 8.00 à 16.00, c'est-à-dire pendant la plus mauvaise partie de la journée pour les processus spectrostadias. Aussi le développement retardait non seulement par cause du jour court, mais plus tard aussi par le manque de prédominance des radiations d'onde longue.

Durée du spectrostade

D'après les travaux de Kouperman (3, 4), Mathon et Stroun (12), Novikov (14), etc., au moment de la gamétogénèse l'intensité de la lumière semble prendre une importance primordiale. Une intensité diminuée cause la stérilité des épis alors qu'une intensité équivalente précédant cette étape n'est pas déterminante. Il ressort de cela que le Spectrostade a lieu au début de la différenciation des épillets (étape IV) jusqu'à la fin de la gonadogénèse (étape V).

Les stades suivants sont encore mal connus. Mais il n'y a pas de doute que des facteurs secondaires au moment du photostade et du spectrostade deviennent dominants plus tard. L'exemple du comportement de la variante *B* (4.00—9.00=16.00—21.00), lors des diverses expériences, dans la suite de son développement est frappant à ce point de vue.

On remarque que, bien que ces plantes aient bénéficié durant le spectrostade de la lumière la plus favorable, elles accusent plus tard un fléchissement dans leur développement (épiaison extrêmement difficile, stérilité forte, fléchissement que ne montrent pas les plantes qui ont bénéficié aussi de la lumière de la première partie du matin mais complétée par celle du milieu du jour: *Série A* (4.00—14.00).

Durées spécifiques du photostade et du spectrostade

Dans l'expérience suivante, nous avons étudié la durée du photostade et celle du spectrostade de deux variétés de Blé soumises à des conditions inadéquates de lumière.

Les variétés de printemps *Alex* (*T. vulgare*), et *Péko* (*T. vulgare*) ont été semées en plein champ le 18.VI.57.

Les plantes se sont développées en 9 1/2 h. d'éclairement (8.00—17.30). De ce fait le développement de toutes ces plantes a été ralenti au cours du photostade par la durée limitée d'éclairement et au cours du spectrostade par le manque de lumière riche en radiations à forte longueur d'onde absentes au milieu de la journée (8.00—17.30).

Par l'étude de la différenciation des cônes de croissance, nous avons pu déterminer chez ces deux variétés le temps nécessaire à l'accomplissement de leur photostade et de leur spectrostade.

Les résultats reportés dans la Figure 11 montrent que:

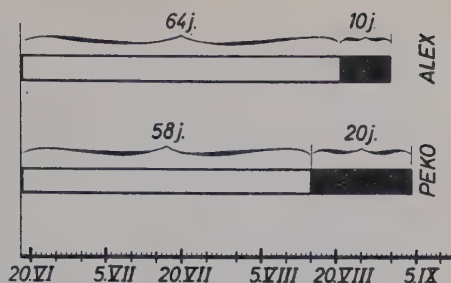
Le durée du photostade de *Péko* (58 jours) est plus courte que celle d'*Alex* (64 jours).

La durée du spectrostade de *Péko* (20 jours) est plus longue que celle d'*Alex* (10 jours).

Figure 11. Durées spécifiques du photostade et du spectrostade.

Blanc: durée du photostade.

Noir: durée du spectrostade



Il résulte de ces constatations que chez une variété l'accomplissement de son photostade peut être *plus long* que chez une autre variété, alors que son spectrostade est *plus court*. Ceci est une nouvelle preuve du caractère mixte de la période considérée. En effet, s'il n'y avait qu'un stade il serait incompréhensible qu'une plante, se développant rapidement sous l'effet de tel facteur, ralentisse soudain son évolution alors que ce même facteur est encore déterminant.

La spécificité des durées du photostade et du spectrostade est d'une grande importance non seulement du point de vue théorique, mais aussi du point de vue pratique:

Hypothèse de travail pour résoudre quelques problèmes pratiques

D'une part nous savons qu'au cours du photostade a lieu la segmentation du cône de croissance à laquelle correspond le nombre d'épillets, et qu'au cours du spectrostade se produit la différenciation des épillets dont dépend le nombre de fleurs.

D'autre part, nous venons de constater que le photostade et le spectrostade sont deux phénomènes spécifiques.

Sur la base de ces faits, nous pensons qu'il serait du plus grand intérêt de faire une analyse stadiale de différentes variétés de céréales, de les croiser et d'étudier le comportement génétique de ces hybrides. A la suite d'un tel travail il ne nous paraît pas impossible que certaines des difficultés relatives aux problèmes de rendement et de précocité puissent être résolues.

Resumé

- 1) La durée des processus photopériodiques — en tant que facteurs déterminants de la floraison — ne s'étend que pendant un stade du développement: le *photostade*.

- 2) Chez les céréales de jour long (Blé, Orge, Seigle), le *photostade* s'étend de la fin de la vernalisation au début de la différenciation des épillets dans le cône de croissance (futur épi).
- 3) Dès le début de différenciation des épillets à la fin de la gonadogénèse, le facteur déterminant est la qualité du spectre lumineux: le *spectrostade*.
- 4) Chez les céréales de jour long les radiations de forte longueur d'onde favorisent les processus du *spectrostade*.
- 5) L'intensité de la lumière est le facteur déterminant au moment de la gamétogénèse.
- 6) La durée du *photostade* et celle du *spectrostade* sont spécifiques.

Summary

- 1) The duration of photoperiodic processes — considered as factors determining flowering — covers only one stage of development: the photostage.
- 2) With long day cereals (wheat, barley, rye), the photostage goes from the end of vernalisation to the moment when the spikelets begin to differentiate in the growth cone (ear to be).
- 3) From the time when spikelets are differentiated to the end of gonadogenesis, the determining factor is the quality of the light spectrum: the spectrostage.
- 4) With long day cereals, long wave length radiations favour the spectrostage process.
- 5) The intensity of light is the determining factor at the time of gametogenesis.
- 6) The respective durations of the photostage and of the spectrostage are specific.

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Investigations on the Mechanism of Absorption and Accumulation of Salts II. Absorption of Phosphate by Potato Tissue

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1. Introduction

It was shown in a previous communication (Lundegårdh 1958 a) that not only the continuously proceeding accumulation of salts but also the rapidly starting initial absorption are both dependent upon the cyanide sensitive fraction of the aerobic respiration, but that the two mechanisms are nevertheless different.

The initial absorption implies charging of the cytoplasmic carriers with anions and cations. Diffusion plays a more subordinate role in this process and it was assumed that the initial absorption is confined to the cytoplasm and implies a loading with salt which may rise up to about 10 times the concentration of the medium. The remarkable sensitivity of the initial absorption to cyanide was attributed to the well-known fact that the undisturbed activity of the cytochrome system is a necessary condition for maintenance of the cytoplasm. It was experimentally shown that only a normally respiring cytoplasmic structure is able to accomplish an efficient initial absorption of salts.

According to the theory of anion respiration the continued accumulation of salts in the sap spaces is conducted directly by the electronic activity of the organized cytochrome system. The process of salt accumulation in the sap spaces is here looked upon as an electrochemical phenomenon.

Even if the electrochemical activity of the cytochrome system is probably

a universal mechanism of salt accumulation other schemes may possibly exist, too (see Lundegårdh 1958 b). One hypothetical mechanism is supposed to be uptake of phosphate by means of reversible phosphorylation. The existence of such a mechanism has never been actually proved. The aim of the present communication is to find out if absorption and accumulation of phosphate in some way deviates from the uptake of chloride.

2. Technique

Potato slices. By means of a microtome potato tissue was sliced in 0.5 mm thick disks. These were cut in pieces of 2—3 mm. side length. The slices were washed in frequently changed distilled water for 2 days. Bubbling air through the medium must be avoided, because the slices then turn brown and lose much of their respiratory activity.

Medium. The experiments were conducted with solutions of KH_2PO_4 , adjusted to pH 6 by addition of K_2HPO_4 .

Chemical procedure. Phosphate was determined colorimetrically with ammonium or vanadium molybdate (blue, resp. yellow color), potassium by means of the Lundegårdh flame photometer. Oxygen consumption was, simultaneously with the salt absorption, determined with the aid of the Winkler method. Also the Warburg respirometer was used for determining the salt effect on respiration. Contrary to wheat roots potato tissue does not evolve disturbing volatile substances.

Fixation of phosphate in organic compounds was determined by parallel analyses of the decrease in phosphate in the medium and rise of inorganic phosphate in the tissue. Contrary to chloride inorganic phosphate is easily washed out by boiling.

The experiments were performed in large water baths with controlled temperature. Pyrex or Quickfit flasks provided with ground stoppers served as containers for objects and solutions (see Lundegårdh 1958 a). The flasks were rotated during the experiments.

3. Experimental results

Absorption experiments were conducted at two widely different temperatures, viz. $+1^\circ$ and $+20^\circ\text{C}$. As shown in Table 1 the initial absorption in the first 15 minutes is only slightly higher at 20° as compared with 1° , a result coinciding with chloride absorption in wheat roots. The later starting active accumulation of phosphate proceeds rather slowly in potato slices and its intensity declines in about $2\frac{1}{2}$ hours from start. Quite in agreement with the previous results on uptake of chloride experiments with renewed solutions show identical figures of active accumulation in one hour and in two hours, if the amount of initial absorption is subtracted from the total absorption in the first hour (see Fig. 1). This result shows that the uptake of

Table 1. *Time course of initial absorption and accumulation of phosphate in potato slices.*
Values in μmol per 1 g fresh weight.

20°C					
KH_2PO_4 M	1	5	15	60	120 minutes
0.005	0.51	1.31	2.61	4.37	5.13
0.010	1.96	3.13	5.60	7.60	8.40
1°C					
0.005	0.75	1.14	2.25	3.29	3.78
0.010	1.42	2.85	4.30	4.90	6.00

Active accumulation between 60 and 120 min.

Temperature	20°C	Temperature	1°C
0.005 M KH_2PO_4	0.76	0.005 M KH_2PO_4	0.49
0.010 M ,,	1.80	0.010 M ,,	1.10

phosphate, too, proceeds in two stages: 1. an initial absorption which is nearly completed in 15 minutes, 2. a period of accumulation starting in 15—30 minutes and running with approximately constant speed for at least a couple of hours.

That different mechanisms are at work in these two phases of salt uptake appears from the obvious differences in the response to a change in the temperature. Table 2 shows a value of Q_{10} for the initial absorption which lies in the region characteristic of physical processes, *e.g.* diffusion and

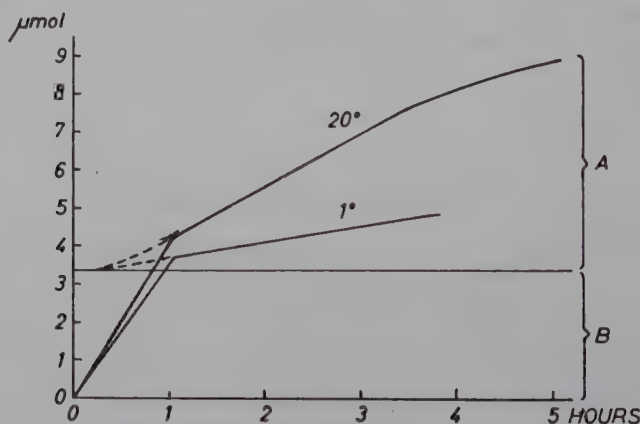


Figure 1. Absorption of phosphate from 0.005 M KH_2PO_4 , at two different temperatures. Values expressed in μmol per h and g fr. wt. The solution was changed at 1, 2½ and 4 hours from the start. The initial absorption thus affects only the first period. The dotted part of the curves represents the calculated start period of continued accumulation.

B=Initial absorption, A=Accumulation.

Table 2. Response of phosphate absorption and cyanide sensitive respiration (AR) in potato slices on changes in the temperature. Values in μmol per 1 g fr. wt. Accumulation per 1 h.

	5°	Q ₁₀	20°
0.010 M KH ₂ PO ₄			
Absorbed PO ₄ initial.....	4.50	1.17	5.60
accumulation	1.09	1.75	2.80
Consumed O ₂	1.14	1.74	3.32
AR	0.69	1.90	2.40
0.010 M KH ₂ PO ₄ + 0.001 M KCN			
Absorbed PO ₄ total	1.39	1.1	1.64
O ₂	0.45	1.50	0.92
0.010 M KH ₂ PO ₄ + 0.005 M KCN			
Absorbed PO ₄ total	—0.74		—0.60
	Q PO ₄ /AR		Q PO ₄ /AR
Total uptake (initial absorption + accumulation)	7.37		3.50
Accumulation only	1.46		1.17

adsorption (ion exchange), *viz.* Q₁₀ = 1.1—1.2, whereas the Q₁₀-values for the period of accumulation are in line with those characteristic of biochemical processes, *viz.* 1.75—1.90.

The fact that the uptake of phosphate is heavily retarded by cyanide does not favour the assumption that phosphorylation acts as a significant mechanism of absorption in potato or roots, because phosphorylases and other phosphate transferring enzymes, *e.g.* hexokinase, are comparatively insensitive to cyanide. Another circumstance speaking against phosphorylation participating in the uptake from moderately concentrated solutions is the fact that only a very small fraction (< 10 %) of the absorbed phosphate is fixed in organic linkage. The initial absorption of phosphate, too, is heavily retarded by cyanide and it is interesting to note that the quantities still absorbed in 0.001 M KCN have a very low temperature coefficient. As in the case of chloride these reduced quantities reflect the subordinate role of diffusion in the initial salt absorption. The experiments, however, point to a somewhat lower sensitivity of phosphate to cyanide than in the case of chloride. The difference is, however, not quite significant, but it can be taken as an indication of some phosphorylation going on. This "extra absorption" of phosphate markedly increases at high concentration, *e.g.* 0.05—0.10 M KH₂PO₄, primarily in roots.

The question as to different sites of adsorption of different anions is still premature, even if it is likely to assume that ions of different shape and size, *e.g.* Cl[−] and H₂PO₄[−], may be attracted by points of different configuration

Table 3. *Sensitivity of H_2PO_4^- and insensitivity of K^+ absorption to cyanide. Values of absorption in $\mu\text{mol/l h. 1 g. fr. wt.}$*

Absorbed ion	0.01 M KH_2PO_4	0.01 M KH_2PO_4 + $3 \cdot 10^{-5}$ M KCN	0.01 M KH_2PO_4 + 10^{-3} M KCN	0.01 M KH_2PO_4 + $3 \cdot 10^{-3}$ M KCN
H_2PO_4^-	7.40	5.25	2.90	0.00
K^+	4.56	4.30	5.25	4.18

in the carrier structure. The only argument speaking in favour of specific anion adsorption is the fact that initially absorbed Cl^- is more firmly bound than initially absorbed H_2PO_4^- . This must, however, not necessarily mean that the sites of adsorption are specific, only that the attachment of one anion may be closer than that of another anion. Dimensions and power of hydration play a considerable role in such circumstances. Against the existence of spatially separated sites of anion adsorption speaks the fact that in potato tissue approximately identical quantities of Cl^- and H_2PO_4^- are adsorbed from solutions of the same concentration.

If the question as to a specific anion adsorption is still undecided the fact that anions and cations are attracted by different sites is on the other hand beyond discussion. It was shown at an early date in this laboratory that cations may be absorbed and exchanged at inhibited anion respiration (see Lundegårdh 1958 b). Table 3 gives a new example of this fact. Addition of increasing amounts of KCN to a solution of 0.010 M KH_2PO_4 slowly chokes the absorption of H_2PO_4^- , whereas the absorption of K^+ is not influenced at all.

4. Concluding Remarks

The main result of the quoted experiments is the fact that at concentrations below 0.020 M phosphate is absorbed and accumulated much in the same way as chloride. It would be expected that if the phosphate anions were to an appreciable extent caught by some special mechanism this would cause deviations as to course and general capacity of the accumulation. Because phosphorylation is the only special mechanism to be thought of in the case of phosphate it can thus be concluded that this process does not appreciably interfere with the common mechanisms of absorption and salt accumulation, at least not in concentrations lower than about 0.02 M.

The only substance in which inorganic phosphate is directly incorporated is glucose-6-phosphate formed by exchange of a glucosidic bond of a polysaccharide for an ester linkage. It would seem that just potato tissue owing

to its high content of starch would be well apted for an accelerated phosphorylation, but the phosphate stored in the washed tissue, amounting to an over-all concentration of 0.001—0.003 *M*, is apparently already optimal for this purpose.

The experiments on uptake of phosphate corroborate and widen the previous results as to the separate mechanisms of initial absorption and accumulation. Significant are here primarily the differences in temperature coefficient. Some further general information about the structural basis of the initial absorption is gathered from a comparison between the absorption of anions and cations.

The experiments show that the structural basis of the primary absorption of anions, *viz.* the anion carriers, is intimately linked to the system of hematin enzymes, whereas the part of the structure adsorbing cations, *viz.* the cation carriers, acts independently of the cyanide sensitive fraction of the respiration. The widely accepted assumption of a strong power of cation adsorption in the living cytoplasm goes back to my work in the period 1928—1941. It was shown at an early date in my laboratory that the living tissues adsorb and exchange cations much in the same way as inorganic colloids, *e.g.* soil colloids, do (Lundegårdh 1931, 1932, Lundegårdh *et al.* 1932, Burström 1934, 1937). These results have been summarized in a separate article (Lundegårdh 1958 b).

The quantitative aspect on the exchange reactions between cations in the medium and cations adsorbed to the negatively charged surface of the cell was further developed in my work 1938—1941 on the surface potentials of roots (see references in Lundegårdh 1958 a). A new oscilloscopic technique enabled accurate measurements of the exchange reactions, to which in a wide range of concentrations the principles of Donnan equilibrium and mass action could be applied. Briggs, Robertson, Hope and others who adopted the idea of the cytoplasm as a predominantly negatively charged colloid reveal, however, a very incomplete knowledge of my earlier work on cation absorption. I have clearly pointed out that what is measured by my rapid technique are just reactions in the surface layer of the cytoplasm. And the measurements here showed an apparent concentration of 10^{-2} to 10^{-4} *M* of the negative sites. I have never claimed, however, that the adsorption of cations is confined to this surface layer of the cytoplasm only. Briggs and Robertson (1957) are here making erroneous interpretations. I have repeatedly emphasized the probable further exchange by means of "carrier rotation" between the surface and the bulk of the cytoplasm.

The total amount of cation carriers in the cytoplasm cannot be calculated from measurements of the surface potential, however, only from adsorbed quantities and these commonly yield higher figures than 10^{-3} *M*. The main

difficulty is here to know exactly the real volume of the cytoplasm in the outgrown cells. If it is hypothetically calculated with 5 % cytoplasm (see Lundegårdh 1958 a) and adsorption of 4—5 $\mu\text{mol K}^+$ per 1 g tissue (Table 3) this means a concentration of cation carriers of ca. 0.1 *M* per litre. Similar values have been calculated by others (see Robertson 1957).

It is here emphasized, however, that a concentration of the cation carriers amounting to 0.1 *M* in the bulk of the protoplasm may very well be combined with a concentration of only 0.001 *M* in the surface layer. Robertson (1957) claims that there is no real surface membrane of the cytoplasm. He believes that the ground substance of the cytoplasm penetrates the inside of the cell wall and that it lets dissolved substances in the medium comparatively freely flow in and out through the cytoplasm. The idea of an interlacement of the cytoplasm in the pectin and cellulose network of the wall is an old one and might in itself imply an acceptable hypothesis, but it was generally assumed by earlier workers discussing these problems that the interwoven part of the cytoplasm is its surface membrane. In denying the existence of a cytoplasmic membrane a tremendous lot of experimental work on permeability and intrability and of microscopic observations on isolated drops of cytoplasm is discarded. I think this is to drive speculation too far. It encounters no difficulties to explain a rapidly proceeding reversible exchange of small ions between the medium and the cytoplasm out from the classical scheme of a protoplast coated by a thin surface membrane. This membrane is namely extremely thin and it already contains a sufficient assortment of ion carriers, of which the negative ones are dominating in number. My measurements of the potential changes accompanying the ion exchange in the membrane show that this process proceeds extremely rapidly. Measurements of slower changes in the potential, *e.g.* at a shift from K^+ to Ca^{+2} and conversely, could be explained from a penetration of the ions into the bulk of the cytoplasm and here initiating processes which recoil into the surface layer. It could from these observations be calculated that the total cytoplasm of the epidermis cells of wheat roots is traversed in very few, in maximum 3 minutes.

The recently discovered fact (Lundegårdh 1958 a) that the anions (Cl^-) and cations (K^+) are initially absorbed in about equal quantities points to a certain balance between cation and anion carriers in the bulk of the cytoplasm even if the cation carriers are dominating in the surface membrane. We are meeting here, however, a very important distinction, namely the fact that the anion carriers are intimately bound to the sites of the cytochrome system and largely lose their function if this system is inhibited, whereas the cation carriers apparently belong to the basic structure of the cytoplasm, which is not disturbed by variations in the activity of the cytochrome system. It may be tentatively assumed that the cation carriers are preferably located

in the hyaloplasm (which also leaves material to the surface membrane) and that the anion carriers are preferably located in the mitochondria.

The observations of the pronounced insensitivity of the cation carriers to inhibitors of the cytochrome system is supported by my earlier observations of the surface potentials of roots. It was shown that the potential, on an average amounting to -60 mv in 0.001 *M* solutions of monovalent salts, remains stationary after a treatment of the roots with cyanide, azide, or fluoride. The surface concentration of cation carriers is thus not influenced by the anion respiration, and the same is now shown for the cation carriers in the bulk of the cytoplasm, too.

Summary

The absorption of phosphate by potato slices is brought about by similar mechanisms as the absorption of chloride by wheat roots. The rapid initial absorption is interpreted as a process of ion exchange and adsorption, proceeding with a very low temperature coefficient. The slower starting continuous accumulation into the sap spaces is quantitatively related to the activity of the cytochrome system. The activity of the cytochrome system also maintains the structural qualities of the cytoplasm which are needed for initial absorption.

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The Inhibitory Effect of Oxygen on the Growth of Wheat Roots

By

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Introduction

Molecular oxygen at pressures above one atmosphere is toxic to living organisms. This oxygen poisoning has been particularly studied in animals and animal tissues. The literature has been reviewed by Stadie *et al.* (1944), Bean (1945), and Dickens (1955). In plants especially the inhibitory effect on respiration of oxygen at considerably increased pressures has been investigated (Caldwell 1956, Turner and Quartley 1956, Quartley and Turner 1957).

As regards germinating and growing plants there are some statements in the literature that even pressures of oxygen about one atmosphere or lower have injurious effects. Thus Kidd (1919) found that the sprouting of potatoes was inhibited in a gas mixture already at oxygen concentrations about 10 per cent of an atmosphere, and 80 per cent oxygen killed the sprouts in 4 to 5 weeks. Albaum *et al.* (1940) soaked oat grains in solutions aerated with different oxygen-nitrogen mixtures and found that 50 and 100 per cent oxygen caused inhibition of the coleoptile growth during the following growth period in air. This oxygen effect was studied further (Albaum 1940, Albaum *et al.* 1942), and it was found to be connected with a decreased translocation of nitrogen compounds from the endosperm to the embryo. Similar inhibitions of the germination have been reported by Barton (1950, 1952, 1953) for several other species. Carbon dioxide protects, according to Barton, the grains

against the injurious effect of oxygen. Regarding the growth of roots there are some statements that pure oxygen at a pressure of about one atmosphere is inhibitory (McPherson 1939, Leonard and Pinckard 1946, Ericson 1946; for older papers, see the review of Clements 1921). However, this effect has evidently not been investigated in detail.

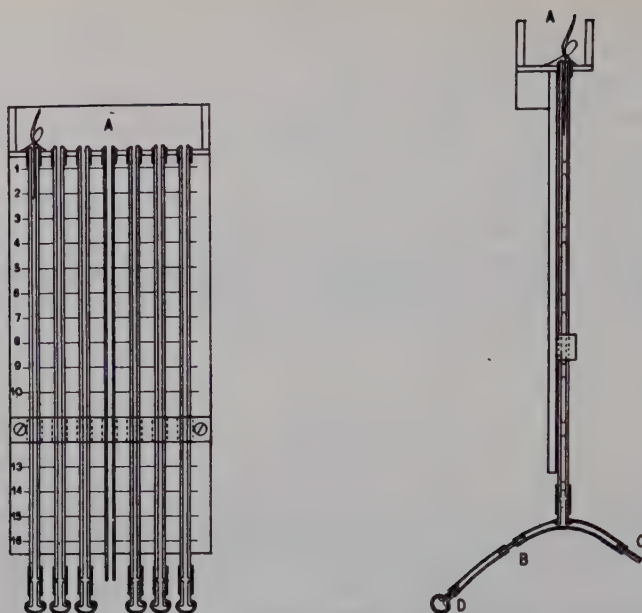
In this paper some experiments regarding the effect of oxygen at a pressure of one atmosphere and lower are reported. They have been performed with a new method permitting the growth of roots in narrow glass tubes. It has been found that oxygen causes an inhibition that develops gradually during the course of one or two days.

Methods

The construction of the experimental device used is shown in Figures 1 and 2. The roots are grown in glass tubes with an inner diameter of about 1.0 mm. The tubes are fastened to a rectangular piece of perspex furnished with a scale in the way shown in the figures. The upper ends of the glass tubes are fixed with rubber tubes in holes in the bottom of a perspex container (A). The seedlings are placed with the main root in the tubes, and solution and air or other gas is forced through the tubes from below. The rate of flow is controlled by the capillary tubes at B and C (Figure 2). The tubes containing the roots are connected in groups of 6 by means of distributors for solution and gas. In the experiments 6 or 12 roots were used for each treatment. The connection from the rubber tube between B and C in Figure 2 to the culture tube goes through a hole in the wall of the rubber tube. The same kind of connection is used for the distributors (D in Figure 2) which are composed of rubber tubes with 6 side tubes in each.

The streaming rate of both the solution and of the gas may be strongly varied without affecting the growth. In the experiments reported here 100 to 200 ml. solution and about four times as great a volume gas have been conducted through each tube per day. After passing the tubes with the roots the solution is drained away from the container A through one of the tubes (the middle one in Figure 1). After the seedlings have been placed in the container A, this is filled with glass beads with a diameter of 2 or 3 millimeters in order to protect the seeds from drying out.

The growth experiments were carried out in the dark in a room with a constant temperature of 22°C. The handling of the plant material and the readings were done in weak light from incandescent lamps. No influence of this light on the growth has been noticed. The experimental material used was Weibull's Eroica wheat. The grains were germinated on moist filter paper in Petri dishes. When the first developed root was about 10 mm. in length, the seedlings were placed with this root in the tubes. After one or two hours the first reading of the root length was made. Usually the growth was determined in air during one day before the treatment with oxygen or some gas mixture of oxygen and nitrogen was started. The oxygen and the gas mixtures used have been obtained in commercial cylinders. As the mixtures with 50 and 70 per cent oxygen caused no or little inhibition, there



Figures 1 and 2. *Diagrams of the culturing device in front and side view respectively. Description in the text.*

is no reason to suspect that the inhibition caused by pure oxygen is due to impurities in the gas. The composition of the nutrient solution used in the experiments except that of Table 2 has been in mmols per litre: Na_2HPO_4 0.1, KH_2PO_4 0.2, $\text{Ca}(\text{NO}_3)_2$ 0.1, MgSO_4 0.05, MnCl_2 0.01, H_3BO_3 0.001, and Fe-citrate 0.001.

The method of growing roots in narrow glass tubes has earlier been used by Gast (1942) and Šeiler (1951). These authors grew maize seedlings in tubes with dripping solution. Audus (1948) used a similar method with circulating solution. Such methods provide several advantages over the usual methods. The device described in this paper makes it possible to supply solution and gas mixtures of fixed compositions continuously. It is easy to change rapidly from one solution or gas mixture to another. As the solution passes the roots only once, the risk of an influence on the growth of changes in the composition of the nutrient solution as a consequence of absorption of nutrient salts by or exudation of organic substances from the roots is eliminated. Exudation of various substances from roots is a common phenomenon especially pronounced during conditions of disturbed metabolism. The literature in this field has recently been reviewed by Frenzel (1957). It is probable that such exudates at least partly may explain the fact that the growth of roots submerged in stationary solutions is less than for roots cultivated in tubes.

Table 1. *Comparison between the growth of wheat roots in tubes and in stationary solutions.* The culture has been carried out simultaneously in the same temperature control chamber (22°C, darkness). Four beakers each with 600 ml. nutrient solution and 48 seedlings on 4 cork holders were used. The solutions were aerated. Each day one holder was removed from each beaker and the length of the first developed root from each seedling was measured. Thus the figures for the root lengths in the beakers are the mean of 48 roots. σ =the dispersion value. Root lengths given in mm.

Root		At start	Days from start			
			1	2	3	4
In tubes	1	13.0	42.0	82.0	117.0	144.0
	2	13.0	42.0	83.5	128.5	162.5
	3	14.0	39.5	79.5	113.0	136.5
	4	15.0	43.0	80.0	113.0	138.0
	5	16.5	45.0	85.0	121.0	146.0
	6	14.5	43.5	75.0	108.5	137.5
Mean		14.3	42.5	80.2	116.8	144.1
In beakers		14.5 \pm 0.2 ($\sigma = \pm$ 1.5)	35.3 \pm 0.7 ($\sigma = \pm$ 4.8)	54.4 \pm 0.8 ($\sigma = \pm$ 5.5)	77.8 \pm 1.5 ($\sigma = \pm$ 10.1)	103 \pm 1.4 ($\sigma = \pm$ 10.0)

Table 1 shows the results of an experiment in which the growth of roots grown on cork disks in beakers has been compared with the growth in tubes. Further, in Table 2 an experiment is presented in which the culture has been made in beakers only. A comparison of the growth values for roots grown in tubes with those grown in stationary solutions shows that there are considerable differences in grown rate between roots grown in stationary solutions and roots growth in tubes, in spite of the fact that the temperature in the experiments has been the same. The fact that carbon powder, a material

Table 2. *The effect of various oxygen pressures on the growth of wheat roots grown in stationary solutions.* Growth at 22°C in the dark in beakers each containing 20 seedlings and 500 ml. nutrient solution of the following composition in mmol/l: KNO₃ 0.2; KH₂PO₄ 0.2, Ca(NO₃)₂ 0.4, MgSO₄ 0.2. pH 5.2. A strong gas stream dispersed by passage through glass filters was bubbled through the solutions. The gas mixtures have been supplied from the beginning of the experiment. Contact between air and solution was prevented by plastic sacks slipped over the beakers. — Average values for 20 to 30 roots. Growth per day in mm.

Oxygen concentration %	Day				Total growth during 4 days (mm.)
	1	2	3	4	
21 air	13	25	26	21	85
5	11	13	15	12	51
50	14	23	26	22	85
100	15	19	11	3	48

capable of absorbing organic substances from the nutrient solution, gives a substantial growth stimulation (Eliasson 1955) is a strong evidence that an inhibitory substance accumulates in stationary growth solutions. This matter will be treated more fully in a later paper.

The method described makes it easy to follow the growth of the roots and to make readings of the root length at short intervals. The growth values presented in this paper give as a rule the growth per 24 hours. The readings of the root lengths have, however, been carried out at considerably shorter intervals, particularly after the beginning of a new treatment. As these values add very little to the picture of the growth course given by the day values they are omitted from the tables. They are, however, used in constructing the time curves of the growth rate and cell lengths for individual roots given in Figures 4 and 5.

Results

The influence of oxygen at a pressure of one atmosphere has been tested in several experiments. The inhibition of the growth in length appears very regularly and always shows the same time course. It begins towards the end of the first 24-hour-period of the oxygen treatment. During the course of the second day there is a strong decrease in growth; and if the oxygen treatment is continued, the growth practically ceases. The time course of the inhibition given by low oxygen concentrations is of quite another type, as is shown by the experiments with 5 and 3.4 per cent oxygen, which for the sake of comparison have been recorded in Table 2 and Figure 3.

The oxygen inhibition obtained with the roots growing in stationary solutions (Table 2) agrees rather well with the one obtained with the tube method

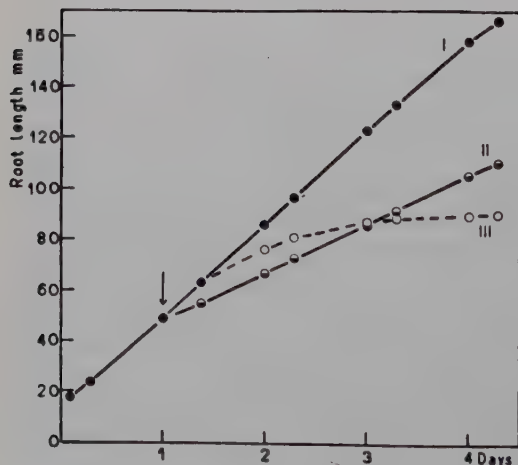


Figure 3. Growth curves for wheat roots in air (I), 3.4 per cent oxygen (II), and 100 per cent oxygen (III). The roots were grown in tubes at 22°C in the dark. The arrow indicates the beginning of treatment. Each curve is the mean for 12 roots.

Table 3. *The effect of 100 and 70 per cent oxygen on root growth.* The first day all the roots have grown in air. The figures give the growth in length in mm. Control and 70 per cent oxygen are average values for 12 roots, 100 per cent oxygen for 6 roots.

Treatment	Day				
	1	2	3	4	5
Control	33	37	31	30	27
100 % O ₂ from 2 nd day	34	28	9	1.5	1.3
70 % O ₂ from 2 nd day	35	36	26	20	17

(Figure 3, Tables 3 to 6). At 70 per cent oxygen the inhibition is only slight (Table 3), and at 50 per cent oxygen there is no inhibition under the experimental conditions used (Table 2). Table 4 shows the reversibility of the oxygen inhibition. If the oxygen is exchanged for air after one day, the roots will soon recover from the rather slight inhibition. If the growth period in oxygen is extended to two days, the growth inhibition will be practically maximal and only a partial recovery will occur if the oxygen is then exchanged for air. A more detailed picture of the recovery is given by the curves in Figure 5.

Root growth is composed of cell multiplication and cell elongation. A possible explanation of the time course of the oxygen inhibition might be that only cell multiplication is inhibited while cell elongation continues unchanged until the earlier formed meristematic cells are used up. In order to investigate this possibility, cell length measurements of the epidermis cells were undertaken mainly according to the methods developed by Burström (see for example Burström 1957). After the completion of the growth experiments, cell lengths have been measured at different distances from the tip. The values obtained have been related to the time curve of the root growth previously recorded. In Figures 4 and 5 cell measurements for 3 control roots and 3 roots grown for two days in oxygen have been recorded. Each point is the mean of 40 to 50 measured cells. The cell number per hour has been obtained by dividing the growth rate by the average cell length. These

Table 4. *The reversibility of the oxygen inhibition.* Italics denote the growth periods in oxygen. The growth values given in mm. per day are means for 12 roots.

Treatment	Day			
	1	2	3	4
Control	32	34	29	22
Oxygen 1 day	32	23	23	21
Oxygen 2 days	38	29	9	6
Oxygen 3 days	36	30	9	1

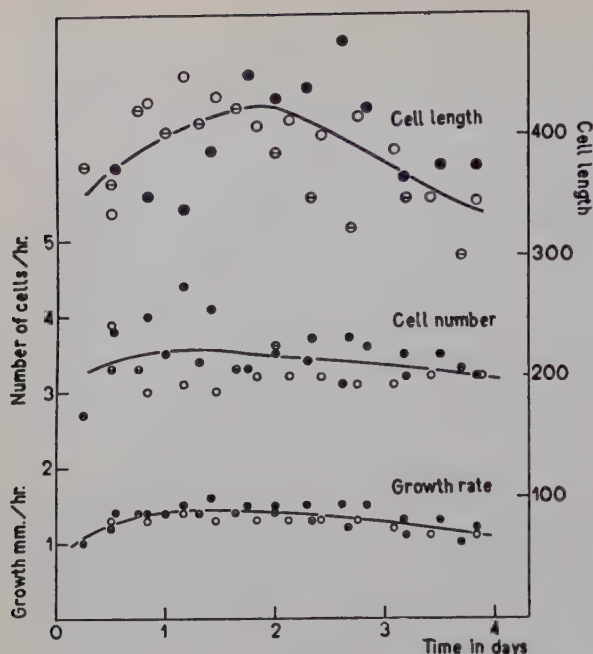


Figure 4. Growth rate, cell lengths, and cell number for 3 average control roots. The points for cell lengths give the average length for 40 to 50 cells in μ . The different roots have been represented with different signs but the curves have been drawn as averages for the 3 roots. Further explanation on page 577.

values thus give the average number of cells in a cell row elongated in one hour. The values of Figure 5 show that the growth decrease in oxygen is connected with inhibition of both cell elongation and cell number. Other cell length measurements on oxygen-inhibited roots gave the same result.

The inhibition of the growth in oat seedlings obtained by Albaum *et al.* (1942) after oxygen treatment of the grains during the soaking might be ascribed to injuries to the enzymes which break down the storage proteins in the endosperm. For this reason some consideration has been paid to the possibility that the growth inhibition might be a consequence of exposure of the endosperms to high oxygen concentrations. As the growth experiments

Table 5. The oxygen inhibition of root growth in light. The second day the culture device with the seedlings was moved to a chamber with strong illumination from fluorescent tubes for 17 hours per day. No precautions were taken to protect the roots against the light. — Average values for 12 roots. Growth in mm. per day.

Treatment	1st day (in the dark 22°C)	2nd day (light 17 hr. 19°C)	3rd day (light 17 hr. 19°C)	4th day (light 17 hr. 19°C)
Control	33	28	22	18
Oxygen from the 2nd day	34	25	11	2

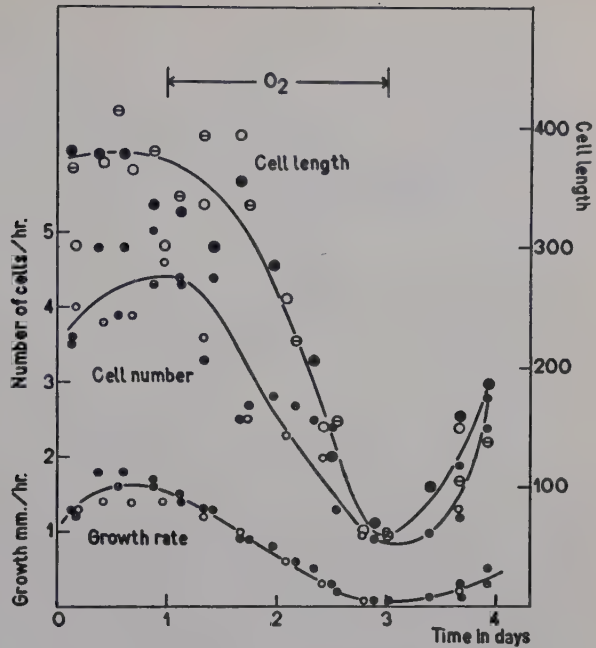


Figure 5. Growth rate cell lengths and cell number for 3 roots grown in oxygen during the second and third day. Explanation on page 577.

were normally carried out in the dark, the seedlings were dependent on the organic substances in the endosperms for growth. One experiment, however, was made in light of such an intensity that the plants were able to carry out photosynthesis. Even in this case oxygen caused inhibition of growth (Table 5). The possibility that the observed oxygen inhibition of the root growth is due to an influence of the oxygen on the endosperm is also contradicted by other experiments. In some preliminary experiments tubes of the type shown in Figure 4 were used. In these the solution and the gas were supplied through the side tube and flowed downwards through the tube with the root. The space around the upper part of the root was filled with agar. Even with this device the typical oxygen inhibition was obtained. Further, the parts of the

Table 6. Growth experiment with 3 roots from the same seedling. The 3 first developed roots from the seedling were put into 3 tubes. Through the tube with the main root air was conducted, through the two others oxygen. Growth values in mm.

Gas	Day		
	1	2	3
Air	35	40	36
Oxygen	30	11	3
Oxygen	32	13	2

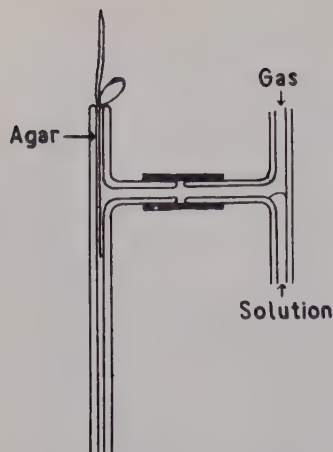


Figure 6. Device for growing roots with supply of solution and gas from a side tube.

plant not exposed to oxygen developed normally. Coleoptiles and leaves of seedlings with oxygen-inhibited roots grew as rapidly as in the control. As for the root growth some experiments have been carried out in which the growth of 2 or 3 roots of the same seedling has been followed in separate tubes. The results from one such experiment are shown in Table 6. The main root has grown in air while the two first developed adventitious roots have grown in oxygen.

Discussion

The fact that the oxygen inhibition develops only some time after the exposure to oxygen has begun may be interpreted in different ways. One possibility is that the effect of oxygen on the growing parts of the root is indirect. The experiments presented above eliminate the possibility that the growth inhibition is a consequence of an oxygen action affecting the processes in the endosperm which are necessary to mobilize the stored nutrients. The possibility that the high oxygen concentration affects the mechanism which transports organic substances through the root must, however, also be considered. Even if this possibility is not very plausible, it cannot at present be excluded.

The most probable interpretation is, however, that oxygen causes injuries to the growing cells. This interpretation is consistent with the obtained results, as such injuries may be developed only gradually. Further, the incomplete and slow reversibility of the inhibition may be due to the fact that these damages are of a rather severe kind.

The nature of injuries caused by oxygen in various organisms has been the subject of extensive investigations. Especially the effect of high oxygen concentrations on enzymes has attracted great interest. The older of these investigations have been reviewed in detail by Stadie *et al.* (1944). Later especially the inactivation of certain enzymes by oxidation of $-SH$ groups has been considered. Barron (1954) has discussed the literature regarding this effect and experimentally tested the sensitivity of such groups to oxidation at various oxygen pressures. Gerschman *et al.* (1954), testing a hypothesis that oxygen poisoning and radiation damages have a common component, obtained a protecting effect against the toxic effect of oxygen in mice by injection of compounds containing $-SH$ groups. These authors are, like Galston and Siegel (1954), of the opinion that the injurious effect of high oxygen concentrations is primarily connected with an increased formation of free radicals and peroxides. Barton and MacNab (1956) found that hydrogen peroxide and to a certain extent also catalase protected seeds of *Phaseolus* against the injurious effect of oxygen bubbling during the soaking period.

Mitochondria *in vitro* have been shown to be very sensitive to molecular oxygen. Thus Hunter *et al.* (1956) found that anaerobic conditions protected the mitochondria from the swelling and loss of oxidative power rapidly occurring in air at 30°C. Kripke and Bever (1956) found that thyroxine protected the mitochondria against the inhibition of certain oxidation mechanisms caused by oxygen bubbling through the mitochondria suspensions. Haugaard *et al.* (1957) studied the toxic effect of oxygen on the enzyme systems in heart muscle homogenate oxidizing glucose and pyruvate.

Considering the facts known about the influence of oxygen on enzymes, there is thus reason to suppose that the oxygen inhibition of root growth is due to an inactivation of an enzyme necessary for growth. For a detailed analysis of the inhibition mechanism further investigations are, however, needed.

The growth inhibition described in this paper was weak at an oxygen pressure of 70 per cent and was lacking at 50 per cent oxygen. With other more sensitive plant materials there may, however, be inhibitory effects of oxygen even at the partial pressure of air. Kidd's results regarding the oxygen sensitivity of sprouting potatoes is an example of this. Further, Albaum *et al.* (1940) found that when the oxygen pressure of the gas bubbled through the solution during the soaking of oats was increased from 2.5 to 20 per cent there was an increase in the growth rate of the coleoptile during the following germination, but the duration of the growth period was decreased to such a degree that the final coleoptile length was smallest at the higher oxygen concentrations used. Similar results were obtained by Brown *et al.* (1952) for the growth of excised segments from the growth zone of *Zea* roots. These authors found that the growth period was considerably longer in 10 per cent oxygen than in air. The fact that Hackett and Schneiderman (1953) did not obtain any effect on growth in excised sections from *Avena* coleoptile or *Pisum* stem by an increase of the oxygen pressure to above two

atmospheres shows, however, that the sensitivity to high oxygen concentrations varies greatly from one tissue to another. There is in any case strong evidence that even the oxygen concentrations occurring under natural conditions in certain cases may have an inhibitory effect on the development of the plants. Besides, as has been pointed out by Malamed (1957), it is the concentration of dissolved oxygen in the solution in contact with the tissue that is of importance. This concentration is dependent on temperature and at unchanged oxygen pressure greatest at the lower temperatures.

Summary

A method for the growing of roots in narrow glass tubes is described. This method makes it easy to follow the growth in length for some days in the beginning of the growth period under controlled conditions. For wheat roots this method gives a greater growth rate than with the roots submerged in nutrient solutions.

The influence of oxygen at a pressure of about one atmosphere on the growth of wheat roots has been investigated. Oxygen has only slight effect on growth during the first day. If the treatment is continued a gradually stronger inhibition is developed and after two or three days the growth has practically ceased. Both the number of elongated cells and cell elongation is inhibited. The recovery is slow and incomplete if the exposure to oxygen has lasted long enough to give strong inhibition.

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Investigations on the Mechanism of Absorption and Accumulation of Salts III. Quantitative Relations between Salt Uptake and Respiration

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1. Introduction

Previous communications (Lundegårdh 1958 b, c) illustrated the occurrence of two phases of salt uptake, namely a short period of initial absorption and a slower starting continued, active accumulation (cf. Figures 1 to 3).¹ Wheat roots and slices of potato tissue are behaving similarly.

The initial absorption is observed, if washed and desalted tissues are transferred to an aerated salt solution. The low temperature coefficient of the initial absorption and the proceeding of the time course (cf. Figure 1) point to adsorption as the main process. Cations are adsorbed more independently of aerobic respiration, whereas a necessary condition for adsorption of anions is the normal proceeding of the cyanide sensitive fraction of the respiration, *viz.* anion or salt respiration. It was concluded that salts are adsorbed to the cytoplasm (including mitochondria) up to about 12 times or more as compared with the concentration of the medium, whereas no or little salt in this short period penetrates the tonoplast. The phase of continued accumulation is characterized by a high temperature coefficient and the salts are in this period accumulated in the sap spaces against the osmotic gradient sap \rightarrow medium.

Much of the confusion prevailing in the literature on salt accumulation

¹ Figs. 1—3 are illustrating new experiments on the time course of salt uptake.

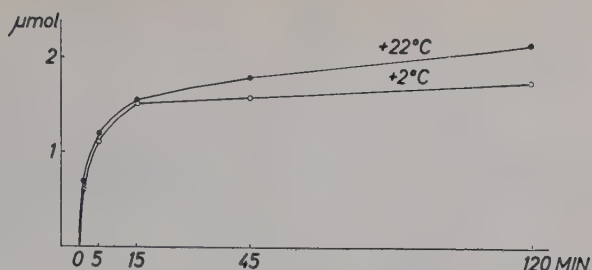
can be brought back to an insufficient knowledge of the start period of initial absorption. It was shown (Lundegårdh 1958 b, c) that the relation between salt uptake and respiration remains approximately quantitative during the first hours of continued accumulation, provided that measurements are starting at the end of the initial period, *viz.* 30 minutes from the start. Higher values of Q_{an}/AR ¹ than 4 were never observed in this period. The cyanide sensitive respiration, on the contrary, does not keep pace with the initial absorption. A sudden burst of respiration is observed in the first minutes of this phase, but it declines; Q_{an}/AR starts with values considerably above 4 and goes rapidly down to figures pertaining to the phase of continued accumulation.

Erroneous interpretations of the quantitative relations between salt accumulation and respiration may be caused by ignorance of the non-specific character of the biochemical process of accumulation. All kinds of anions and cations are accumulated, but the speed varies according to electrophoretic mobility and power of adsorption. Of fundamental importance is the fact that also movable organic anions produced in the aerobic metabolism are caught by the same mechanism. A co-variation between the internal concentration of malate and the intensity of the anion respiration has been observed (see Lundegårdh 1958 d), and a number of observations indicate a competitive attraction to the mechanism of accumulation, causing less salts being absorbed from the medium at high production of organic acids, and *vice versa*. Too little attention has unfortunately been paid to this competitive transport and accumulation, the knowledge of which, however, is fundamentally important for the appropriate pretreatment and handling of the experimental objects and for interpretation of Q_{an}/AR , in which "an" stands for *imported* anions, not for anions circulating in the tissue (see Lundegårdh 1954, 1955, 1958 d).

About ten years ago a new approach was made to the problem of quantitative relations between the imported salt anions and the respiration (Lundegårdh 1949 b, table 1 and 2) namely the effect of gradually increasing concentrations of KCN. These experiments showed approximately constant values of Q_{an}/AR . The calculations were here made from the *total* salt absorption, *viz.* initial absorption+accumulation during 2 hours. In the light of the recent elucidation of the two phases of absorption this result would mean that initial absorption and continued accumulation are both equally affected by cyanide. In view of the importance of this conclusion for picturing the possible mechanism of salt uptake a corroboration of the previous results was considered desirable.

¹ an=imported anions, AR=fraction of the total respiration inhibited by 0.001 M KCN. The ground respiration remains unchanged.

Figure 1. Time course of the absorption of chloride by washed potato slices. 0.002 M KCl. Two temperatures, showing the different response of initial absorption and continued accumulation. On the ordinate $\mu\text{mol Cl}$ absorbed per g. fr. wt.



The experiments were performed with wheat roots and potato slices. The experimental technique has been described in the communications 1958 b and c. The experiments lasted 2 hours. Cyanide (KCN) was added in five concentrations from 10^{-5} to 10^{-3} M.

In order to illustrate the competitive effect of imported anions on anions circulating in the tissue new experiments were also conducted with tissues washed in extended periods and with salt solutions of different concentration.

2. Experimental Results

a. Approximate constancy of Q_{an}/AR at increasing inhibition by cyanide

Tables 1—2 show the results of experiments on the absorption of chloride by potato slices or wheat roots. Table 3 shows experiments on the absorption of phosphate by wheat roots.

The absorption of anions from diluted salt solutions (0.002—0.005 M) is largely (see below) inhibited in the presence of 10^{-3} M KCN.

As shown by the approximate constancy of Q_{an}/AR the retardation of the cyanide sensitive respiration is keeping pace with the simultaneously retarded salt absorption. In the experiments with phosphate (Table 3) a drop

Table 1. *Potato slices*. Chloride absorption and O_2 -consumption at increasing additions of KCN. Values in μmol per 1 h. 1 g. fr. wt. Q_{Cl}/AR calculated from observed respiration minus respiration in 10^{-3} M KCN (=AR). Basic Cl-concentration 0.005 M KCl. Seven parallels. 20°C.

Activity measured	Concentration of added KCN M					
	Controls	10^{-5}	$3 \cdot 10^{-5}$	10^{-4}	$3 \cdot 10^{-4}$	10^{-3}
Absorbed chloride ...	2.59	2.11	1.34	0.95	0.51	0.00
Consumed oxygen ...	2.59	2.30	1.76	1.53	1.24	0.90
AR.....	1.69	1.40	0.86	0.63	0.34	0.00
Q_{Cl}/AR	1.53	1.51	1.56	1.51	1.50	—

Table 2. *Wheat roots*. Chloride absorption and O_2 -consumption at increasing additions of KCN. Values in μmol . per 1 h. 1 g. fr. wt. The anion respiration (AR) is calculated from observed respiration minus respiration in 10^{-3} KCN. $Q\text{ Cl/AR} = \frac{\text{absorbed Cl}}{\text{anion respiration}} \cdot 20^\circ\text{C}$.

KCl M	Controls	$+10^{-5}$ M	$+3 \cdot 10^{-5}$ M	$+10^{-4}$ M	$+3 \cdot 10^{-4}$ M	$+10^{-3}$ M KCN
<i>Chloride absorption</i>						
0.002	1.22	1.06	0.62	0.44	0.21	0.07
0.005	3.25	2.85	2.35	1.48	0.77	0.08
<i>Oxygen consumption</i>						
0.002	6.12	5.85	5.46	5.09	4.60	4.27
0.005	6.42	6.00	5.62	5.16	4.76	4.28
<i>Anion respiration</i>						
0.002	1.85	1.60	1.17	0.82	0.33	0.00
0.005	2.14	1.70	1.35	0.90	0.42	0.00
<i>Q Cl/AR</i>						
0.002	0.66	0.70	0.52	0.54	0.64	—
0.005	1.52	1.68	1.74	1.65	1.83	—

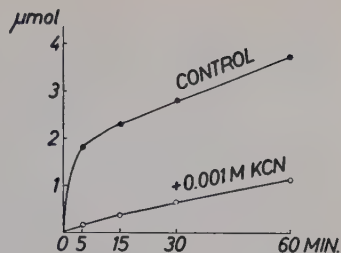
of about 10 % is observed between the controls and the first stage of inhibition ($+10^{-5}$ M KCN), but the difference is hardly significant. If phosphate were to any appreciable extent absorbed by a separate mechanism (see Lundegårdh 1958 c) the values of $Q\text{ an/AR}$ would on the contrary rise with increasing cyanide inhibition.

The initial absorption of chloride in potato slices amounts to about $1.5\text{ }\mu\text{mol Cl}$ in 0.002 M KCl and about $2.5\text{ }\mu\text{mol Cl}$ in 0.005 M KCl (see Figures 1 and 2). In experiments lasting two hours the uptake of chloride in the period of accumulation (15—120 minutes) comprises only about 15—40 % of the absorbed total quantities. *The approximately constant relation between absorbed quantities of salts and the intensity of the cyanide sensitive respiration is consequently equally valid for the initial absorption and the continued accumulation.*

Table 3. *Wheat roots*. Phosphate absorption and O_2 -consumption at increasing addition of KCN. Values in μmol . per 1 h. 1 g. fr. wt. $\text{AR} = \text{anion respiration}$. 20°C . $0.005\text{ M KH}_2\text{PO}_4$.

Activity measured	Controls	$+10^{-5}$	$+3 \cdot 10^{-5}$	$+3 \cdot 10^{-4}$	$+10^{-3}$ M KCN
H_2PO_4	2.86	1.91	1.36	0.44	0.00
O_2	6.59	6.40	6.28	5.39	5.13
AR	1.46	1.27	1.15	0.26	—
$Q\text{ H}_2\text{PO}_4/\text{AR}$	2.17	1.98	1.81	1.89	—

Figure 2. Time course of the absorption of chloride from 0.005 M KCl and 0.005 M KCl+0.001 M KCN. Washed potato slices. 1°C. On the ordinate $\mu\text{mol Cl}$ absorbed per g. fr. wt.



As mentioned in a previous communication (Lundegårdh 1958 c) anions may to a certain extent enter the tissues also in solutions, to which 10^{-3} M KCN was added. Figure 2 gives an additional example. The time course deviates, however, completely from that observed in pure solutions of chloride, chiefly because the characteristic period of initial absorption is very much reduced or fails to appear. With 10^{-3} M cyanide the chloride anions are simply flowing in at a slow approximately constant speed.

b. The effect of varying salt concentration and prolonged washing

New experiments confirmed the observation (see Lundegårdh 1958 b, Table 5) that at 20°C . the total absorption of chloride in 2 hours rises approximately in line with the concentration of the medium up to about 0.010 M KCl (see also Lundegårdh 1949 a). It was pointed out that the initial absorption here serves as the pacemaker of active accumulation. Anions are apparently fed into the mechanism of accumulation *via* the initial absorption, the intensity of which is steeply rising with the concentration. The controlling influence of initial absorption is shown also by the experiments in section a, with the difference that in this case initial absorption and accumulation are both equally inhibited.

In low concentrations of a pure salt the anion respiration is reduced too, but to a lesser extent than the initial absorption, depending upon the fact that now anions circulating in the tissue are taking over relatively more of the activation of the cytochrome system. At increasing import of salts these circulating anions, to which also malate and other organic acids belong, are competitively expelled from the surroundings of the cytochrome system.

Owing to the high Q_{10} -value of the anion respiration (Lundegårdh 1958 b, Table 5) imported and initially absorbed anions share a smaller part of the total assembly of anions in the surroundings of the cytochrome system when the temperature is high than when it is low. This is the reason why at the same salt concentrations Q_{an}/AR of continued accumulation rises to much

Table 4. *Potato slices*. Influence of extended washing in distilled water on the efficiency of salt accumulation. Values in $\mu\text{mol. per 1 h. 1 g. fr. wt. } 20^{\circ}\text{C. } 0.005 \text{ } M \text{ KCl} + 10^{-4} \text{ } M \text{ KCN.}$

Time of washing	1 day	2 days	4 days
Cl-absorption	1.38	1.46	1.81
Anion respiration (AR)	1.02	0.84	0.77
Q Cl/AR... ..	1.30	1.74	2.35

higher values at 3° than at 21° C. and may finally touch the "critical figure" 4, even if no cases were observed, in which this value was significantly surpassed.

The competitive effect of imported anions on the free circulating anions may be enhanced by prolonged washing, *viz.* by removal of circulating salts. In the experiments quoted in Table 4 the anion respiration was reduced about 50 % by addition of $10^{-4} \text{ } M \text{ KCN}$. The table shows that tissues washed 4 days are taking up relatively more chloride than tissues washed only 1 day. And this in spite of the fact that the anion respiration owing to the added KCN is reduced by 50 %. Similar results were obtained without addition of KCN. The rising efficiency of the anion respiration may be interpreted as the result of sinking competition with circulating anions.

In experiments with wheat roots, which were washed in distilled water for 8 days, values of Q an/AR very close to 4 were observed. These values are of course calculated for *the phase of continued accumulation* (30—120 minutes). Calculations based on the *total* salt absorption, *viz.* initial+accumulation, would here give values exceeding 4, from which, however, no reliable conclusions as to the efficiency of the mechanism of active accumulation can be drawn,

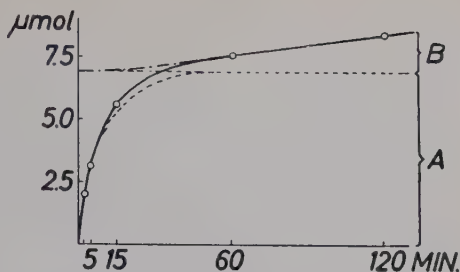
3. Discussion ¹

a. Interpretation of observed quantitative relations

One main result of the experiments is the fact that the initial absorption and the continuous accumulation behave differently toward temperature and variations of the concentration but are both dependent on the activity of the cytochrome system and to an equal degree inhibited by cyanide. Because in experiments lasting 2 hours the initial absorption amounts to quantities which as a rule lie above 50 % of the total absorption (see Figures 1 to 3) any deviating response to cyanide would inevitably have caused a drift in Q an/AR

¹ Pertaining to communications I—III.

Figure 3. Absorption of phosphate by washed potato slices, 0.010 M potassium phosphate. Dashed curves: calculated course of initial absorption and continued accumulation. 1°C. On the ordinate $\mu\text{mol PO}_4$ absorbed per g. fr. wt. A=Initial absorption, B=Accumulation.



at increasing inhibition. A slight drift may be observed in Tables 2 and 3, which could possibly indicate some variation in the response of the two phases of salt absorption to cyanide. With due allowance made for inevitable biological variations at random the differences cannot be regarded significant, however.

The strong effect of 10^{-3} M KCN on the initial absorption of chloride appears from the fact that only 20 % of the total volume of the tissue is invaded in 1 hour. The controls without cyanide are absorbing this quantity of chloride in about 2 minutes (see Figure 2).

Effects similar to those appearing from a treatment with cyanide are observed on treatments with dinitrophenol and fluoride, inhibitors which are interfering with the electron transference through the cytochrome system, too (Lundegårdh 1949 b, 1958 d). Common to all disturbances of the transport of electrons from dehydrogenases to oxygen is the fact that most of the oxydative phosphorylation is impeded. Oxydative phosphorylation is needed for synthetic work in the protoplasm. A causal relation between salt absorption and high energy phosphate metabolism has been suggested by a number of investigators (Robertson, Wilkins and Weeks 1951, Overstreet and Jacobsen 1952, Sutcliffe and Hackett 1957 and others), but no serious attempt has been made to picture an acceptable scheme of how such a mechanism would work.

A stoichiometric relation between absorbed anions and produced ATP (adenosine triphosphate) would amount to about 6—8 anions per consumed O_2 (a vast number of biochemical experiments yielded maximum values of P/O between 3 and 4). According to the equilibrium $\text{ADP (adenosine diphosphate)} + \text{PO}_4 \xrightleftharpoons[2]{1} \text{ATP (adenosine triphosphate)}$ one phosphate anion disappears for each ATP formed (reaction 1). The remaining cation, *e.g.* K^+ , would then be ready to combine with one imported anion, *e.g.* Cl^- . If reaction 1 takes place near the surface of the mitochondria and the reacting salts are adsorbed to carriers it would no doubt facilitate the entrance of anions from the surrounding cytoplasm.

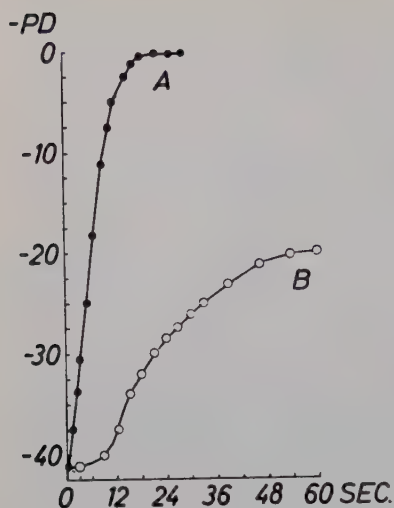


Figure 4. Time course of the ion exchange in the surface of wheat roots, measured as changes in potential by means of a rapid oscillographic method (Lundegårdh 1941). A=living root; 0 sec. indicates the change from 10^{-3} to 10^{-1} KCL. B=root killed in alcohol or hot water.

The hypothesis of a causal linkage between phosphorylation and salt uptake yielding Q_{an/O_2} values above 4 may be experimentally examined in different ways.

Merely the fact that the mentioned inhibitors of oxydative phosphorylation also inhibit salt absorption is in itself no argument supporting the hypothesis because all inhibitors affect the electron transference in the cytochrome system and, according to the theory of anion respiration, an undisturbed transport of electrons *through the whole system* is needed for salt accumulation. As to the magnitude of the quotient an/O_2 it has never been conclusively shown that *the process of active accumulation into the sap spaces* yields values significantly above 4. Values of ≤ 4 are more in line with the theory of anion respiration. High values regularly appear in *the phase of initial absorption*. They start, however, considerably *above* 6—8 in the first minutes and decrease rapidly toward the end of the initial phase.

Table 4 in the first communication of the present series (Lundegårdh 1958 b) shows values of $\frac{\text{absorbed anions}}{\text{consumed } O_2}$ amounting to 20.5 in the first five minutes and 13.2 in 15 minutes. Even if allowance is made for a somewhat higher probable error in the readings from the first period (5 minutes) these results clearly show that the velocity of the initial absorption far exceeds the value 6—8 pertaining to an hypothetical linkage to oxydative phosphorylation. As a matter of fact the time course of initial absorption *closely follows that of adsorption to a substrate, not that of a continuously running*

process (cf. Figure 4, in which the exchange adsorption in the surface layer of root epidermis is pictured).

A strong *argument against phosphorylation* serving as a mechanism of *initial salt absorption* is the low Q_{10} value of this process. The activity of the *cytochrome system*, which is reflected in the *anion respiration*, shows Q_{10} -values far above those observed in the *initial absorption* (see also Fig. 1). And available experience teaches us that biochemical Q_{10} -values are valid for oxydative phosphorylation, too.

Against the hypothesis of phosphorylation as a mechanism of salt absorption is thus speaking: (1) the low Q_{10} -values of the initial absorption, and (2) the experimentally determined Q_{an/O_2} -values (≤ 4) of the active accumulation into the sap spaces, which are more in accord with the theory of anion respiration.

The theory of anion respiration thus still offers a plausible scheme of active accumulation. *The initial absorption*, and, from a wider point of view, *all processes connected to carrier functions*, such as adsorption and ion exchange, *must be treated as separate mechanisms which are feeding ions into the mechanism of active accumulation*.

b. Properties of the cytoplasmic structure

Even if we know that the osmotic work involved in raising the salt level of the sap spaces is intimately connected to the activity of the cytochrome system several important problems are still unsolved, however. One of these, which is accessible to experimental examination, is the structural basis of the process. We know that the cytochrome system is carried by "mitochondria" or "particles" in the cytoplasm. These particles may be collected in homogenates. We know that these particles show a certain power of salt absorption (Robertson 1957) and respond upon this absorption with an increased O_2 -consumption. These observations are very similar to the burst of cyanide sensitive respiration observed in the first stage of initial absorption. The results of Robertson may be explained from a similar viewpoint as the results of Bartley and Davies (1954), *viz.*, an exchange absorption steered by the Donnan equilibrium. This is no accumulation comparable to that in a sap space which is devoid of immobile large carrier ions (R^+ and R^-).

The vast literature on "mitochondria" (see Millerd and Bonner 1953, Green and Beinert 1955) deals primarily with the biochemical properties of sediments from homogenates, but little is known about the identity of the "mitochondria fractions" with structures in the living cytoplasm. In a recent investigation on homogenates from wheat roots (Lundegårdh 1958 a) I have found that all fractions, sedimented at accelerations of 3800 g. to 20.000 g.

at increasing time intervals contain *the cytochromes a_3 and b in about the same proportions*. This means that large portions of *wheat cytoplasm are provided with a cytochrome system*. Observed variations as to the other cytochromes, *viz.*, c , c_1 , and b_3 , are due to the fact that these cytochromes, primarily c , are more or less detached during the process of mild homogenization (grinding 15 minutes at 0° in $0.3\text{--}0.5\text{ }M$ glucose + $0.02\text{ }M$ phosphate). In the mentioned investigation an attempt was made to determine the quantity of cytochrome carrying substance.

1000 mg. fresh roots yield 30 mg. total cytoplasmic sediment, containing $0.23\text{--}0.27$ mg. cytochrome b and corresponding quantities of $a + a_3$, *etc.* These 3 % of "structure" is not very far from what may be calculated as the percentage of cytoplasm in outgrown roots (Lundegårdh 1958 b).

100 mg. dried roots (6 % of the fresh weight) contain about 50 mg. salts + sugar (calculated from the osmotic value = ca. 6 atm.), and for the rest cell walls and cytoplasm (the volume of the nucleus is here negligible). The sedimented structures of the cytoplasm comprise 16 to 17 % of the total dry substance, of which about 40 % refers to cell walls. The structures contain only about 33 % water (= "bound water") in a medium of 0.3 to 0.5 M glucose. This figure tallies with the idea that the living cytoplasm contains comparatively little water.

These calculations show that the conception mitochondria is of little value in the present situation. What we are discussing here are properties of the basic part of the structure of the cytoplasm. This structure is more or less broken down to particles during grinding. Some of these particles may correspond to real mitochondria others are certainly debris of the basic structure of the cytoplasm. The surprising fact that the structure-bound cytochromes are so uniformly distributed in all fractions (Lundegårdh 1958 a, Table 1) points to a specific spatial arrangement, *e.g.*, as mono- or multi-molecular layers near to or coating the surface, or as submicroscopic units which may be torn apart during the homogenization. It may be mentioned in connexion with these results that Green (1957) in homogenates of animal origin has found particles of much smaller size than the mitochondrion, *viz.*, dimensions similar to microsomal particles. They show the capacity of catalyzing the oxidation of succinate or diphosphopyridine nucleotide by oxygen.

Since it has now been shown that the overwhelming part of what is called "cytoplasmic structure" must be figured participating in salt absorption and accumulation the problem of active transference of salts to the sap space *via* the tonoplast appears in a new light. Microscopic observations show streaming of strings, filaments and more isodiametric elements of the visible structure sliding along the tonoplast. The active cytoplasm is practically coating the

sap room, from which it is separated only by the thin tonoplast. Cytochrome systems are thus in direct contact with the tonoplast and may here exert their salt pumping quality. It is not necessary to assume a stationary layer of cytochrome systems at the boundary between the cytoplasm and the tonoplast. The submicro entities may be movable in the streaming cytoplasm and turn their activity into unidirectional salt transport just in the moments of close contact.

The susceptibility of the cytoplasmic structure to inhibitors, *e.g.* malonate (own observations), and cations, *e.g.* K^+ and Ca^{+2} (on the latter see Florell 1956), is a well known fact. The same may be said of the "endoplasmic reticulum" of animal cells (Palade 1956). It is also likely to assume that the properties of the structure depend on the $ADP \rightleftharpoons ATP$ balance, which is easily moved in one direction or the other owing to variations in the supply of ATP (Lundegårdh 1955 a, Eliasson and Mathiessen 1956). All these influences are most probably affecting the carrier functions and the exchange capacity of the structures.

Bartley and Davies (1954) in their study of ion transport by kidney mitochondria claim that these behave as mixtures of cation and anion exchange resins and that metabolic energy might be needed to generate or maintain the carrier functions, *viz.* the immobile non-diffusable R^+ and R^- complexes. This conception is very similar to that one resulting from our own research work, but Bartley and Davies did not carry on the analysis of the process of salt absorption as far as is done here. They believe that, according to the Donnan equilibrium, the carrier complexes may be able to "maintain high gradients of both cations and anions in salt solution". As I have repeatedly emphasized in my previous work the Donnan equilibrium in itself, together with adsorption and ion exchange, cannot serve as a mechanism for accumulation of free salts. It can only raise the level of concentration at local sites, and thus create a more favorable start situation to the real process of accumulation in the cell sap, which is a mechanism requiring incessant expenditure of energy. Overstreet (1957), too, inadequately believes that the process of absorption to the carrier structure is identical with true accumulation.

4. Conclusions

The main results of the communications I—III may be summarized as follows:

In *washed and desalted tissues* the salts are entering the cytoplasm *via* carriers (R^+ and R^-) in the surface membrane of the cytoplasm. This process runs extremely rapidly (Figure 4). By means of "carrier rotation" or

"adsorption tracks" or both the ions move into the bulk of the cytoplasm. It was assumed that the salts (anions+cations) are, by means of exchange processes and according to the Donnan principle, "adsorbed" to the "structures" ("mitochondria" etc.). This is *the phase of initial absorption*, which in the investigated objects attained a steady state in about 15 minutes. The combined adsorption of cations and anions raises the level of concentration considerably above that of the external medium. Because salts stimulate the activity of the cytochrome system the initial absorption mediates a rapidly passing burst of cyanide sensitive respiration. This wave of stimulated activity is no doubt promoting an increased carrier capacity, thus a kind of autocatalysis. The phenomenon, too, sheds light upon scattered biochemical reports on the importance of an appropriate salt level for maintaining a high metabolic activity.

The process of initial absorption is in itself non-metabolic, viz. needs no extra supply of energy, but *the carrier functions of the cytoplasmic structure must be metabolically maintained*, viz. they need a continued activity of the cytochrome system and concomitantly a positive $\text{ADP} \rightleftharpoons \text{ATP}$ balance.

In the case of *unwashed tissues*, which are taken directly from nutrient solutions or cut from intact storage organs, the initial absorption is already a more or less stationary phenomenon, viz. the carrier structure is charged with salts circulating in the tissue. New imported salts are in this case compelled to compete with the stationary ions before they are caught by the mechanism of accumulation.

The initially absorbed salts represent the level of concentration from which the continued accumulation is starting, a fact reflected in the difference of the start lags. This appears from the fact that no direct relation between the velocity of salt accumulation and the external concentration may be observed at low temperature. At higher temperature, when the accumulation is the pacemaker, such a relation may be observed.

The essential difference between the process of initial absorption and the process of continued accumulation appears from the fact that the first one involves a non-metabolic adsorption *into* the structure, whereas the second one conveys a metabolically steered transport *from* the structure to the sap space. Special carriers or "tracks" may cooperate in this process but it is an undisputed fact that in all closer studied cases the cytochrome system was shown to play a dominant role.

The ambiguity of the conception "free space" is still more emphasized by our new findings. If the structural basis of initial absorption and accumulation is extended to the major part of the visible cytoplasm only a minor part is left to really "free water", which may be in direct communication with the comparatively larger quantities circulating in the cell walls. If the con-

ception "free space" ought to be applied to the cytoplasmic structures is a matter of choice. The adsorbed salts, which comprise the overwhelming part of the initially absorbed ones, are certainly not "free" as compared with a solution without carriers. But they are more or less exchangeable and a steady state, steered by the Donnan equilibrium etc., prevails between the adsorbed ions and the really "free" salts in the intermicellar water of the cytoplasm, the intermicellar water of the walls (which have a very low power of adsorption; cf. Figure 4), and the external solution. But we are then simply turning back to previous conceptions of "non-metabolic absorption" and "exchange capacity", conceptions which are better in line with our actual knowledge.

5. Summary

Two main phases may be distinguished in the uptake of salts by washed tissues:

(1) A rapidly accomplished initial absorption. (2) A slower starting continued accumulation.

Both processes are equally sensitive to disturbances in the transference of electrons through the cytochrome system, but they are reflecting two different sides of its activity and show characteristic differences as to temperature ratios and response to varying conditions.

Chloride and phosphate may be absorbed and accumulated by the same mechanism.

Cations and anions are absorbed by different carrier systems.

The bearing of the results on hypotheses as to the mechanism of active salt accumulation is discussed.

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A Separation of Auxin-Induced Cell Wall Loosening into its Plastic and Elastic Components

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An auxin-induced increase in plasticity of *Avena* coleoptile cell walls was first demonstrated by Heyn in 1931 (8). In Heyn's experiments, as in those of subsequent investigators such as Söding (17) and Thimann (19), a small amount of expansion was allowed to take place between the time of application of auxin and the measurement of plasticity. This prevented differentiation between the two sequences: Auxin action \rightarrow increased plasticity \rightarrow growth, and Auxin action \rightarrow growth \rightarrow increased plasticity. Cleland and Bonner (5) used the expansion of coleoptile sections in water containing an inhibitor of auxin action to measure the loosening of the cell wall induced by a previous incubation of the sections with auxin in a slightly hypertonic mannitol solution. Since this technique allows the action of auxin to be completely separated in time from any expansion, the increase in length of the auxin-treated sections over the non-auxin-treated controls indicated that the sequence is Auxin action \rightarrow cell wall loosening \rightarrow growth. This cell wall loosening can be due to an increased plasticity, increased elasticity, or a combination of the two. The purpose of the present work is to determine the contribution of each of these components to the auxin-induced cell wall loosening.

Two methods have been most widely used for this purpose. The first, used by Heyn in his classic experiments and subsequently improved by Tagawa and Bonner (18), involves measuring the reversible and irreversible angles of bending produced in a horizontally held coleoptile by the addition of a small weight to the unsupported end. Objection has been raised by Pohl (14) to relating the reversible and irreversible angles of bending to, respectively, elasticity and plasticity since the deformations produced by the weight must, in the absence of water uptake, not only occur in the longitudinal but also in the transverse direction. The ease of transverse movements of water in the tissue will also affect the results obtained by this method. In any case, the increase in irreversible bending must be due in part to an increased stretching of the longitudinal cell walls on the upper side of the coleoptile.

A second method for separating elasticity and plasticity has been developed by Ursprung and Blum (20), who measured by plasmolysis of the tissue before and after the expansion the increase in plastic and elastic stretching which occurred during expansion of the tissues in water at 0°C. A modification of this technique has been used in these experiments. Anaerobiosis rather than low temperature is used to prevent auxin action during the expansion. Since plasmolysis results in some damage to *Avena* coleoptile cells so that the growth rate is decreased (9), at the beginning of the expansion period only one portion of the sections are plasmolyzed and the rest are allowed to expand. These are then plasmolyzed and the lengths compared with the controls to determine the amount of plastic stretching. This plastic stretching, however, is only a measure of the expansion that has occurred and is not necessarily a measure of the potential plasticity that existed. Thus an increase in the osmotic pressure of the tissue may result in an increase in plastic stretching without a change in plasticity occurring. Stiffening of the tissues which has been shown to occur by several workers (1, 5, 8, 16) results in a decrease in the contraction of the cell walls during plasmolysis. This cannot be distinguished from plastic stretching.

Since neither method alone gives unambiguous results, it was decided that both methods should be employed. A comparison of the two results, then, might indicate the exact nature of the cell wall loosening which is induced in water saturated tissues by optimal auxin concentrations. The evidence of Pohl (15) suggests that this reaction is not identical with the reaction that occurs in naturally growing coleoptiles under the influence of only endogenous auxin. This possibility has not been examined in this paper.

The use of the term plasticity as a synonym for irreversible deformability of a tissue has been generally used since the work of Heyn (8). It should be realized, however, that this is undoubtedly incorrect usage in a strict physical sense. Moreover, it should be realized that plasticity is a measure of the

potential and not the amount of irreversible deformation. In this paper, plastic is used as a synonym for irreversible and the terms plastic bending and plastic stretching are used to indicate the actual processes. The term plasticity is only used to indicate the potential for plastic stretching. The term elasticity has been treated in the same manner.

Methods

Bending Experiments

The procedure is identical with that of Tagawa and Bonner and makes use of the bending machine described in their paper (18). *Avena* coleoptiles were grown in the manner of McRae and Bonner (11). Coleoptiles of 3.3 cm. length were selected. The central leaf was removed and one 2.0 cm. section was then cut 3 mm. from the tip. The sections were floated for one hour in 10 ml. of 0.25 or 0.28 *M* mannitol solution either containing or lacking IAA (5 mg./l.). There was no increase in length of the sections during this period. The coleoptiles were then secured to the bending machine by insertion of a stainless steel pin of about 1 mm. diameter and 5 mm. length into the empty leaf chamber at the basal end of the coleoptile section. A fine glass rod, weighing 75 mg. and 5 cm. in length, bearing a central loop, was then inserted into the tip end of the coleoptile to a depth of 5 mm. The tip of this glass rod projected upon a protractor. After 5 minutes, the initial angle was read. A weight was then suspended from the loop (225 or 100 mg.). After 15 minutes the angle was again read. The weight was then removed and after 5 minutes the final angle was determined. The difference between the initial angle and the final angle

Table 1. *Plastic and elastic deformation induced by subjecting coleoptile sections to an imposed load for 15 minutes. Coleoptiles treated with 0.28 M mannitol for one hour, \pm IAA, 5 mg./l. Plastic and elastic angles of deformation then determined as explained in text.*

Auxin	Amount of deformation: angle in degrees	
	Elastic	Plastic
+	13	29
+	16	29
+	18	27
+	14	26
+	11	20
—	17	16
—	19	13
—	10	11
—	14	10
—	11	9
Average: +	14 ± 1.4	26 ± 1.8
—	14 ± 1.9	12 ± 1.4
Difference	0 ± 2.4	14 ± 2.3

is the plastic bending. The difference between the final angle and the total angle induced by the weight is the elastic bending.

All measurements were carried out in a dark room under a dim red light at 25°C. and at almost 100 % humidity. The time periods used for the various operations have been determined experimentally by Tagawa as suitable for the purpose.

It is possible to get some idea of the reproducibility of the system from the data in Table 1 where the values for the plastic and elastic bending are given for five pairs of coleoptiles. It is evident that variations of greater than ± 5 degrees cannot reasonably be expected to be chance variations.

Expansion Experiments

The procedure used is a modification of that of Cleland and Bonner (5). Coleoptiles were grown in the manner of Fransson and Ingestad (7). When the coleoptiles had reached a length of 2.75—3.25 cm., a 5 mm. section was cut from the tip of each coleoptile after the apical 2 mm. had been discarded. Lots of twenty sections were used for each portion of each experiment. One lot was measured and then plasmolyzed by immersion for 90 minutes in 1 *M* mannitol. The length was then remeasured under a microscope with eyepiece micrometer. The remaining sections underwent three successive treatments. During an auxin pretreatment period the sections were floated for one hour in 10 ml. of 0.28 *M* mannitol buffered with 0.0025 *M* K-maleate, pH 4.8, and either with or without added indoleacetic acid, 5 mg./l. This concentration of mannitol was such that at the end of the one hour the length of the sections was slightly less than the initial length. The sections were then allowed to expand in 10 ml. of buffered water containing CO₂-free N₂. After 90 to 120 minutes the sections were removed and measured. They were then plasmolyzed in 1 *M* mannitol and remeasured.

The elastic stretching (E) of the tissues is calculated by subtracting the average plasmolyzed length of the tissues after expansion from the length on removal from the expansion solution and before plasmolysis. The amount of plastic stretching (P) is found by subtracting the plasmolyzed length before treatment from the plasmolyzed length after expansion. The differences between auxin and non-auxin treated tissues are expressed as ΔE and ΔP values. A positive value indicates an increase due to the addition of auxin. All values are expressed in ocular units where one unit equals 0.0625 mm.

Standard errors of difference for the plastic and elastic stretching of auxin and non-auxin treated sections have been calculated and are about 0.25 units for most experiments. Thus a difference in ΔE or ΔP of 0.5 units is considered significant at the 5 % level. This is illustrated in Table 5.

The experiments reported in this paper were carried out in green light. Similar experiments carried out in red light have given the same qualitative results.

Results

Bending Experiments

Five experiments were performed using a total of 22 coleoptiles treated with auxin and an equal number of non-auxin treated control coleoptiles. The

Table 2. *Average plastic and elastic angles of deformation induced by an imposed load for 15 minutes as influenced by pretreatment with auxin in slightly hypertonic mannitol solution. Coleoptile sections treated for one hour in 0.25 or 0.28 M mannitol solution, \pm IAA, 5 mg./l. Plastic and elastic angles of bending then determined as explained in text.*

Coleoptile pairs	Mannitol conc. M	Weight	Angles in degrees	
			ΔE	ΔP
3	0.25	225 mg.	+1	+13
6	0.25	100	+4	+8
5	0.28	100	0	+14
6	0.28	100	+3	+9
2	0.28	100	+1	+10

Table 3. *Angles involved in measurement of plastic and elastic angles of bending for one pair of coleoptiles. Conditions same as in Table 1. Angles in degrees.*

Mode of Bending	Auxin	
	+	—
Angle: Initial	68	73
+ weight	23	40
Final	41	57
Total Bending	45	33
Plastic Angle	27	16
Elastic Angle	18	17

Table 4. *Reversal of the auxin-induced increase in plastic bending by cyanide ions. Coleoptiles are incubated in 0.28 M mannitol, \pm IAA, 5 mg./l., for 15 minutes. Then sufficient KCN is added to bring concentration to 3×10^{-4} M. After 45 minutes angles of bending are determined as explained in text.*

No. of Coleoptiles	Auxin	Angles in degrees			
		E	ΔE	P	ΔP
5	+	22	0	40	+1
5	—	22		39	
6	+	22	0	30	—2
6	—	22		32	

results are summarized in Table 2. It can be seen that the values are in reasonable agreement and certainly indicate a definite effect of auxin upon the plastic bending and probably a lesser effect upon the elastic bending. Table 3 gives the actual angles involved for one pair of coleoptiles.

The presence of 3×10^{-4} M KCN after incubation with auxin completely reverses the auxin-induced cell wall loosening. The effect of KCN on the

auxin-induced plastic bending has been determined by incubating the sections with or without IAA in hypertonic mannitol solution and after 15 minutes adding sufficient KCN to bring the concentration to $3 \times 10^{-4} M$. The sections are removed from the solution at the end of an hour and the angles are determined. The results, given in Table 4, show that the effect of the auxin pretreatment on the plastic bending is completely reversed by the cyanide ions.

Expansion Experiments

A resolution of the auxin-induced cell wall loosening into its plastic and elastic components is shown in Table 5. An increase in the irreversible stretching of the sections due to an earlier auxin pretreatment is obtained when sections are allowed to expand under anaerobic conditions. The increase due to auxin treatment is not as great in these experiments as in those reported earlier (5) due to a decrease in the growth rate of sections in response to auxin.

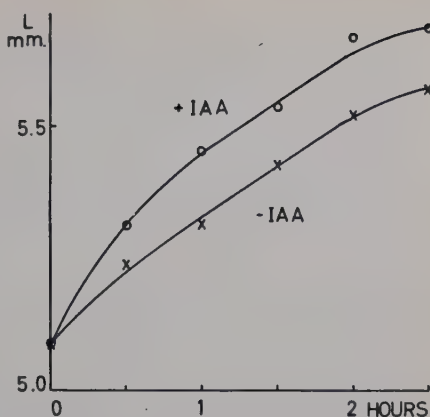
Table 5. *Resolution of cell wall loosening into plastic and elastic stretching.* Sections pre-treated with 0.28 M mannitol, \pm IAA, 5 mg./l., 1 hour. Expansion in water, $+$ N₂, 2 hours. Plasmolysis for 90 minutes in 1 M mannitol. Initial length after plasmolysis: 70.5 ± 0.13 units.

Auxin	Final length		Lengths in ocular units			
	Before plasmolysis	After plasmolysis	P	ΔP	E	ΔE
+	82.5 ± 0.14	73.8 ± 0.14	2.4 ± 0.19	$+0.8 \pm 0.26$	8.7 ± 0.20	0 ± 0.25
—	81.7 ± 0.09	73.0 ± 0.14	1.6 ± 0.17		8.7 ± 0.14	

Table 6. *Effect of cyanide and auxin during expansion period on plastic stretching.* First period, one hour; second period, two hours. Plasmolysis 90 minutes in 1 M mannitol.

Conditions		Auxin in 1st period	Lengths in ocular units			
1st period	2nd period		P	ΔP	E	ΔE
a) water	none	+	1.7	$+0.7$	9.9	$+0.2$
		—	1.0		9.7	
b) 0.25 M Mannitol	N ₂ , water	+	3.6	$+0.6$	9.3	$+0.4$
		—	3.0		8.9	
c) Mannitol	$3 \times 10^{-4} M$ KCN, water	+	2.5	-0.1	8.8	$+0.1$
		—	2.6		8.7	
d) Mannitol	N ₂ , water	+	3.5	$+0.6$	8.8	$+1.2$
		—	2.9		7.6	
e) Mannitol	N ₂ , water, IAA	+	3.5	$+0.8$	9.1	$+1.3$
		—	2.7		7.8	

Figure 1. Time curve of expansion of sections in oxygen-free water after pretreatment for one hour in 0.25 M mannitol, \pm IAA, 5 mg./l. No auxin in expansion period solution.



The increase in potential for plastic stretching induced in one hour by auxin in non-expanding tissues is a large fraction of the auxin-induced increase in plastic stretching which occurs during one hour of expansion in water, as is shown in lines a and b in Table 6. This is further illustrated by Figures 1 and 2. If after incubation in slightly hypertonic solution, with or without auxin, the sections are allowed to expand in oxygen-free water, no equilibrium in length is reached but a constant and equal rate of expansion for both sets is obtained after the first hour. During the first hour, however, the auxin treated sections show an increase in expansion due to their prior auxin treatment.

It proved to be unnecessary to have a treatment containing both mannitol and N_2 between the auxin treatment and the expansion in order to block the auxin action before the expansion. This is indicated by the fact that addition of IAA to the expansion period solution elicited no additional expansion (Table 6).

The effect of auxin upon the elastic stretching is variable. This can be

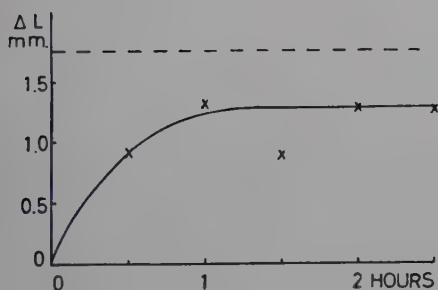


Figure 2. Increase in length of auxin pretreated sections over non-auxin treated controls as a function of time in oxygen-free water. Sections pretreated for one hour in 0.25 M mannitol, \pm IAA, 5 mg./l. Expansion in water bubbled with N_2 , —IAA. Dotted line is amount of auxin-induced growth of sections during one hour in water, + IAA.

seen by comparing the figures for elastic stretching in Tables 5 and 6. While there is no increase in the elastic stretching due to auxin in one case, in the other the magnitude of the auxin-induced elastic stretching is greater than that of the plastic stretching.

In the presence of 3×10^{-4} M KCN, cell wall loosening can be reversed. This reversal affects both the elastic and the plastic components (Table 6).

Discussion

The influence of auxin upon coleoptile sections in a hypertonic solution has been investigated by determining the reversible and irreversible angles of bending produced by application of a weight to the unsupported end of horizontally held coleoptiles. The addition of auxin causes a large increase in the irreversible or plastic bending. There is probably a smaller increase in the reversible or elastic bending although the later effect is too small to be statistically certain. The increase in plastic bending can be completely abolished by treatment of the sections with 3×10^{-4} M KCN after the auxin pretreatment. Thus auxin changes the properties of the sections in some way so that the sections have a greater potential for irreversible deformation, and this increase in potential can be reversed by cyanide ions. These results are in agreement with those of Heyn (8).

An auxin-induced loosening of the *Avena* coleoptile cell wall has been demonstrated by Kobayashi *et al.* (10) and Cleland and Bonner (5). This loosening has now been separated into its plastic and elastic components by means of a modification of the technique originated by Ursprung and Blum (20). The presence of auxin results in an increase in the plastic stretching which is constant and predictable. The presence of cyanide ions abolishes this increase.

Elastic stretching shows a variable response to the presence of added auxin. In some experiments there is no increase in elastic stretching in the presence of auxin and in others the increase in elastic stretching is greater than in plastic stretching. It is believed that this variation is a function of the condition of the coleoptiles before cutting since similar results are always obtained for each treatment within any one experiment but not between experiments. This problem must be examined more closely.

There can be several possible causes of the increased plastic bending or the increased plastic stretching due to auxin. Only an increase in irreversible extensibility or an increase in permeability can explain both cases, however. The determinations of Ordin (12) and Ketellapper (9) on the permeability

of *Avena* coleoptile sections to heavy water as influenced by auxin have shown that auxin causes no increase in permeability to water. Burström and Fransson (4) investigated the growth of water saturated coleoptiles and concluded that permeability could not cause the growth. Thus unless there is a different mechanism for each of the two processes, or unless both are due to artifacts inherent in the techniques used, auxin must be causing an increase in the potential for irreversible stretching of the walls as shown both before the expansion (bending) and afterwards (expansion). Although increased intussusception of new cell wall material has been shown to occur during auxin-induced growth of *Avena* coleoptiles (3) and pea stems (6), the studies of Bonner on growth at low temperatures (2) and the C-14 glucose incorporation studies of Ordin *et al.* (13) indicate that this increase in intussusception occurs in response to the increased expansion and not to auxin itself. The potential for irreversible stretching, then, must be a potential for plastic extension of the wall already present.

The magnitude of the increase in plastic stretching induced in one hour by auxin in non-expanding tissues is from 50—80 percent of the auxin-induced increase in length which occurs during one hour of growth of sections in water. It is not surprising that the amount of expansion produced by a certain length of auxin treatment is less when the potential must be conserved for a period of time than when it is used up immediately. Such would be in agreement with the results of Pohl (16) and Adamson and Adamson (1) which indicate that a loss of auxin effect with time or a stiffening process must occur. Thus it appears that the majority if not all of the increase in length which is elicited by auxin in tissues in water is of the nature indicated by these experiments.

Normal auxin-induced growth of a tissue depends upon more factors than just an increase in wall plasticity. Intussusception of new cell wall material, osmoregulation, and availability of water are certainly necessary for continued growth. These factors, however, do not appear to be directly influenced by auxin but, rather, are influenced by the magnitude of the auxin-induced expansion. Only the cell wall plasticity is affected by auxin in the absence of any expansion.

Growth may occur in the *Avena* coleoptile, then, in the following manner. Auxin causes an increase in the plasticity of the wall which initiates the expansion. A continual process of cell wall loosening followed by expansions results in continued expansion as long as there is, among other things, intussusception of new cell wall material, osmoregulation, and water available for expansion. Extension growth is the sum of all these processes but only the loosening of the cell wall is auxin dependent.

Summary

1. The auxin-induced loosening of the cell wall has been separated into its two components, plastic and elastic extensibility by two different techniques.
2. A determination has been made of the reversible and irreversible angles of bending which occur in horizontally held coleoptile sections in response to added weight at the unsupported end, after incubation of the tissues in hypertonic mannitol solution with or without IAA. The presence of auxin causes a large increase in the plastic bending and possibly a small increase in the elastic bending.
3. A determination has been made of the plastic stretching of coleoptile sections by measuring the increase in plasmolyzed length of tissues after treatment with auxin in slightly hypertonic mannitol solution and then expansion of the tissues in water. The elastic stretching is measured by comparing the lengths of plasmolyzed and unplasmolyzed tissues after the expansion in water. Auxin causes a consistent increase in the plastic stretching. The effect on elastic stretching varies from none in some experiments to an amount greater than that for plastic stretching in others.
4. Both plastic bending and plastic stretching are reversed by 3×10^{-4} M KCN, as is growth.
5. The increase in growth produced in water saturated coleoptiles by addition of IAA in a concentration of 5 mg./l. appears to be primarily due to an increase in plastic extensibility of the walls already present.

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The Occurrence of Acid Inhibitors in Resting Terminal Buds of *Fraxinus*

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It has earlier been shown (Hemberg, 1949 b) that growth-inhibiting substances occur in resting terminal buds of *Fraxinus*. These substances are found in the outer brown-coloured bud scales as well as in the inner parts of the buds. The inhibitors disappear from the buds when their rest is broken, regardless of whether this process takes place naturally or is brought about by treatment with ethylene chlorhydrin. Hemberg (1946, 1947, 1949 a, 1950) has also demonstrated growth-inhibiting substances in the peel of resting potato. Also here the substances disappear in connection with the breaking of the rest period. In the potato peel there are partly acid and partly neutral inhibitors (Hemberg, 1949 a). It is, however, only the acid inhibitors that disappear in connection with the termination of the rest period (Hemberg, 1952).

Blommaert (1955) demonstrated an acid inhibitory substance in resting peach buds. This had on chromatographing in *n*-butanol saturated with 2 N ammonia Rf values between 0.5 and 0.9. The substance was evidently inhibitor β (Bennet-Clark and Kefford, 1953). Blommaert showed that the amount of this inhibitor gradually decreased as the rest became less and less deep. When the rest period was completely broken, the substance had entirely disappeared. Also Hendershott and Bailey (1955) found inhibitors in resting peach buds and showed that these disappeared in connection with the termination of the rest period. Additional literature on the occurrence of growth-inhibiting substances and their role in the rest period of different plant organs may be obtained from the reviews of Hemberg (in press 1 and 2).

The aim of the present investigation has been to determine whether the acid inhibitors in ash buds disappear in connection with the termination of the rest period as they do in peach buds.

Material and Methods

The material used in this investigation was terminal buds from the same specimen of *Fraxinus excelsior* L. f. *pendula* that was studied in the previously published report (Hemberg, 1949 b). The buds were collected on two occasions, on October 28, 1957 when the buds were resting and on February 7, 1958 when the rest period must have been over. Each time 50 buds were collected. Extractions were made of the two outermost pairs of decussate bud scales, hereafter called peripheral bud parts, and also of the rest of the buds, hereafter called central bud parts. Peroxide-free ether was used for the extractions, which were carried out for 45 hours at $+2^{\circ}\text{C}$. The ether was changed three times during the course of the extractions. The extracts were separated into acid and neutral fractions. Due to lack of time only the acid fraction, which should contain eventually occurring acid inhibitors, was examined.

The extracts were chromatographed in isopropanol—ammonia (sp. wt. 0.91)—water (100:14:6) on Whatman paper no. 1. The chromatograms were developed and tested biologically in the same manner as previously described (Hemberg, 1958 a), however, with two deviations. Firstly, wheat coleoptiles (variety Ergo 2 from A.B. Weibulls, Landskrona) were employed as test plants instead of oat coleoptiles and, secondly, for the biological assay the pieces of the chromatograms were placed in small beakers containing 2.0 ml. citrate buffer instead of 4.0 ml. previously used.

Results and Discussion

The results, which are evident from Figure 1, show that at the time of the first extraction, when the buds were in deep rest, acid growth inhibitors were found in extracts of the peripheral as well as of the central bud parts. The strongest inhibition in the chromatograms occurred between the R_f values 0.5 and 0.7 (see Figure 1 A and B), *i.e.*, at the place where one may expect to find inhibitor β . In addition a suggestion of inhibition was detectable at lower and higher R_f values (ca. 0.2 and 0.9 respectively). The extract amounts that were transferred to the chromatogram papers were intentionally very small in order that maximal inhibition should not be reached in the test experiments. This would have made the detection of an eventual decrease in the amount of inhibitory substances more difficult.

In the extracts that were prepared on February 7, 1958 of buds, which for the most part in any case must have left the rest period, considerably

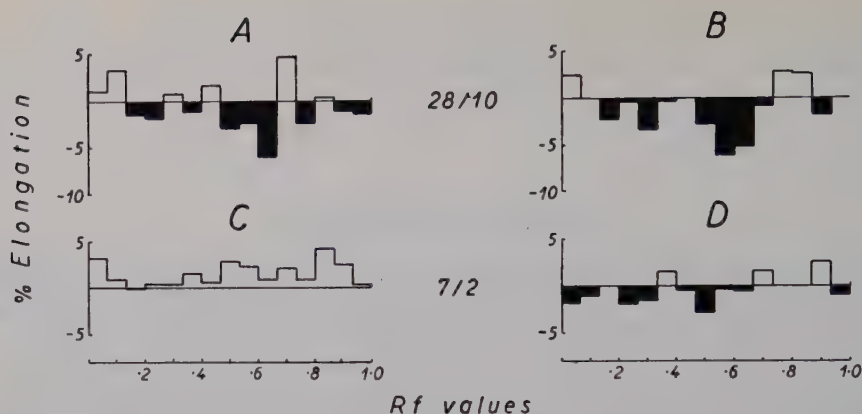


Figure 1. *Biological determination of chromatograms of acid fractions of extracts of peripheral (A and C) and central (B and D) bud parts of terminal buds of ash. A and B refer to extracts prepared on October 28, 1957, C and D to extracts prepared on February 7, 1958. Extracts from 0.05 g. plant material in A and C and from 0.10 g. in B and D. Abscissa: Position of the paper segment on the chromatogram in Rf units. Ordinate: Growth of wheat coleoptile segments in per cent of growth of the control segments.*

smaller amounts of inhibitory substances could be detected (see Figure 1 C and D). In the extract of peripheral bud parts no traces whatsoever of inhibitors could be found. In the chromatogram of the extract of the central bud parts there was a weak inhibition at the place for inhibitor β , however considerably weaker than in the chromatogram of the extract from October 28, and a suggestion of inhibition between the Rf values 0.0 and 0.4.

It is thus evident that inhibitor β occurs in demonstrable amounts in resting ash buds and that this inhibitor disappears during the course of the winter from the buds. The results obtained agree well with what Hemberg (1949 b) earlier found on inhibitory substances as regulators of the rest period of ash buds. They also support Blommaert's (1955) discovery that acid inhibitors play a role in the regulation of the rest period in peach buds. Moreover, inhibitor β appears to regulate the rest also in other plant organs. Thus experiments of Blommaert (1954), Varga and Ferenczy (1956 and 1957) and Hemberg (1958 b) show that this inhibitor is found in the peel of resting potato but it disappears from the peel when the rest is broken.

Judging from the histograms reproduced in Figure 1 there are only very small demonstrable amounts of auxin in the buds, neither in those extracted in October nor in those extracted in February. This agrees with what Blommaert (1955) has shown. He did not find any auxin in resting peach buds. Auxin could be demonstrated in such buds first in connection with the shooting of the buds.

Summary

In resting terminal buds of *Fraxinus excelsior* L. f. *pendula* there is an acid inhibitor, which judging from the R_f value on chromatographing in isopropanol—ammonia—water is inhibitor β . This inhibitor, which occurs in resting buds in the two outermost pairs of decussate bud scales (peripheral bud parts) as well as in the central parts of the buds (central bud parts), disappears from the buds when these during the winter leave the rest period naturally.

This investigation is carried out partly with contributions from the Swedish Science Research Council. The wheat employed in the test experiments has been supplied by A.B. Weibull, Landskrona, through the courtesy of Fil. dr. O. Gelin. The author wishes to thank Mrs. Maija-Liisa Holmberg and Mrs. Janina Pisarska for their valuable technical assistance.

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The Significance of the Inhibitor β Complex in the Rest Period of the Potato Tuber

By

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(Received April 30, 1958)

Introduction

In earlier works it has been shown (Hemberg, 1946, 1947, 1949, 1950) that ether-soluble growth-inhibiting substances occur in the peripheral parts, the so-called peel, of resting potato. The substances disappear when the potato leaves the rest period naturally or as a result of treatment with ethylene chlorhydrin. Hemberg (1949) has likewise shown that these inhibitors comprise partly acids and partly neutral substances. It is only the acid inhibitors that disappear in connection with the termination of the rest period, whereas the neutral ones, which occur in varying amounts in different potato varieties, may even increase in amount toward the end of the rest period (Hemberg, 1952). The central parts of the potato tuber contain considerably smaller amounts of acid inhibitors than the peel layer (Hemberg, 1954).

Blommaert (1954) and Varga and Ferenczy (1956, 1957 a) have with the help of paper chromatography demonstrated the occurrence of an acid inhibitory substance in the peel of resting potato. Judging from the chromatographic results it is inhibitor β (*cf.* Bennet-Clark and Kefford, 1953). They find in conformity with Hemberg that the acid inhibitor disappears when the potato leaves the rest period naturally or through treatment with "rindite" (a mixture of ethylene chlorhydrin, ethylene dichloride and carbon tetrachloride, 7:3:1 by volume). According to Varga (1957 b) inhibitor β is

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composed of a complex of organic acids, among others derivatives of salicylic and cinnamic acids.

Burton (1956), who examined six different potato varieties, contrary to the previously cited investigators, could not find any correlation between the occurrence of ether-soluble inhibitors in the potato peel and the rest of the potato tuber. Burton also studied the inhibitor content of the peel extracts with the help of paper chromatography. However, he did not fractionate the extracts into neutral and acid fractions prior to the chromatographing but chromatographed the crude extracts with water as the chromatographing fluid. The chromatograms were thereafter divided into two zones, which were examined separately by means of the *Avena* straight growth test. The one zone, which Burton called the "promoting zone", comprised that part of the chromatograms which had R_f values between 0.3 and 0.65. It contained growth-stimulating substances, evidently neutral auxins (*cf.* Linskens, 1955, p. 154). The other zone was called by Burton the "inhibiting zone". It comprised that part of the chromatograms which had R_f values between 0.65 and 1.0. According to Burton this zone contained the acid inhibitors. At the same time it also contained indole acetic acid, which is cursorily mentioned in the paper. The R_f value of this acid in water is 0.89 (Linskens, 1955, p. 154). Indole acetic acid comprises a large part of the natural auxin of the potato (Hemberg, 1947; Varga and Ferenczy, 1956, 1957 a). The presence of this substance in the "inhibiting zone" must have affected the findings in the biological test. Since, according to Hemberg (1952), different potato varieties can contain unequally large amounts of acid auxin and the auxin content of the different varieties does not have any connection with the duration of the rest period of the respective variety, the indole acetic acid in the "inhibiting zone" must exercise varyingly strong influence in experiments with the different potato varieties. Moreover it is possible that the neutral inhibitors also occurred in the "inhibiting zone", since Burton does not mention that he found inhibitors in another place in the chromatograms. The neutral inhibitors occur in varying amounts in the different potato varieties, and they do not disappear, as already stated, in connection with the termination of the rest period.

Burton pointed out that he considered it less suitable to determine the inhibitor content in extracts of potato peel by means of the *Avena* test, as earlier investigators have done, and from this draw conclusions on the role of these inhibitors in the rest of the potato. Instead one should employ as the test material the plant whose rest one wishes to study. He tried therefore to use potato shoots as the experimental material for the demonstration of the inhibitors. This method, however, proved to be unsuitable. Against Burton's reasoning it can be said that if the *Avena* method cannot be used for demonstrating the presence of inhibitors in different plant organs, it should not either be applicable for demonstrating the occurrence of auxins in

different plant organs. We know, however, that the same auxins are found in many different species and this also holds true for inhibitors. Thus inhibitor β has been found not only in the potato tuber but also in other plant organs (*cf.* Blommaert, 1955; Köves, 1957; Varga, 1957 a and b; Varga and Ferenczy, 1957 b; Hemberg, 1958 b; for further literature see Hemberg, *in press* 1). One should, of course, therefore, be able to make use of *Avena* coleoptiles or any other suitable experimental material whatsoever for demonstrating the presence or absence of this inhibitor complex. Then if one wishes from the results obtained to draw conclusions concerning, for example, the role of the demonstrated inhibitors in the rest of a plant organ, one should, of course, know something about the duration of the rest period of this organ. This can, however, be easily obtained by carrying out sprouting experiments.

The reader is referred to the review of Hemberg (*in press* 2) for additional literature concerning the rest of the potato and other plant organs.

Material and Methods

Three different potato varieties with unequally long rest periods have been studied, namely Eigenheimer, Magnum bonum and Majestic. Eigenheimer is known to have a very short, Magnum bonum a medium long and Majestic a very long rest period. Both Eigenheimer and Majestic were not completely ripe at harvesting due to the cold rainy summer in 1957. Their rest period was therefore unusually long (*cf.* Emilsson, 1949, p. 208). On the other hand, the rest period was normal for Magnum bonum, which was harvested somewhat later than the two other varieties and had had time to ripen completely before harvesting. The potato was cultivated at the Institute for Plant Research and Cold Storage in Nynäshamn. It was stored there after harvesting in a dark, air-conditioned storage room, the temperature of which was never below $+4^{\circ}\text{C}$. Each time an extract was to be prepared, potatoes were taken from this storage room, transported to Stockholm and prepared for extraction not more than six hours after they had been taken out of the storage room.

The potato peel was extracted with peroxide-free ether at $+2^{\circ}\text{C}$ for 45 hours in the same manner as in the earlier described experiments (Hemberg, 1952). The extracts were fractionated into acid and neutral fractions. Only the acid fraction was subjected to testing. This fraction was chromatographed in isopropanol—ammonia (sp. wt. 0.91)—water (100 : 14 : 6) on Whatman paper no. 1. The chromatograms were examined biologically in the same manner as previously described (Hemberg, 1958 a) but with wheat instead of oat coleoptiles.

At the time of each extraction sprouting experiments were also started. In these 15 potatoes of each variety were planted in boxes with a layer of wet sand at the bottom. The potatoes were half buried in the sand, the boxes covered with glass and placed in a dark room at $+20^{\circ}\text{C}$. In order to prevent the penetration of illuminating gas from adjacent laboratories, which would have an injurious effect on the course of the sprouting, fresh air was continuously blown into the room from outside. Fourteen days after the planting the length of appearing sprouts was measured. If several sprouts had grown from one potato, the length of the longest one was measured as well as the combined length of all sprouts longer than 2 mm.

By these sprouting experiments it was shown that *Eigenheimer* and *Magnum bonum* under the experimental conditions in question could indeed sprout somewhat even during the earlier stage of the rest period, but that the growth of the sprouts during this period was considerably inhibited. The experiments with extraction and planting of potatoes were continued until the growth-inhibiting substances in the potato peel were practically no longer demonstrable in chromatograms of the peel extracts.

Results

Eigenheimer. This potato was harvested on September 13, 1957. The first extract was prepared on September 19. At the same time sprouting experiments with 15 potatoes were started. On October 3, i.e., 14 days later, 12 of these potatoes had sprouted with one sprout each. The lengths of the sprouts on the different potatoes were the following: 15, 10, 7, 7, 6, 6, 5, 4, 4, 4, 2 and 2 mm. The inhibition of sprouting evidently varied in the different potatoes. The seven potatoes which had sprouts at least 5 mm long were freed from these and peeled, and the peel was extracted with ether. The inhibitor content in extracts of peel from unsprouted potatoes, prepared on September

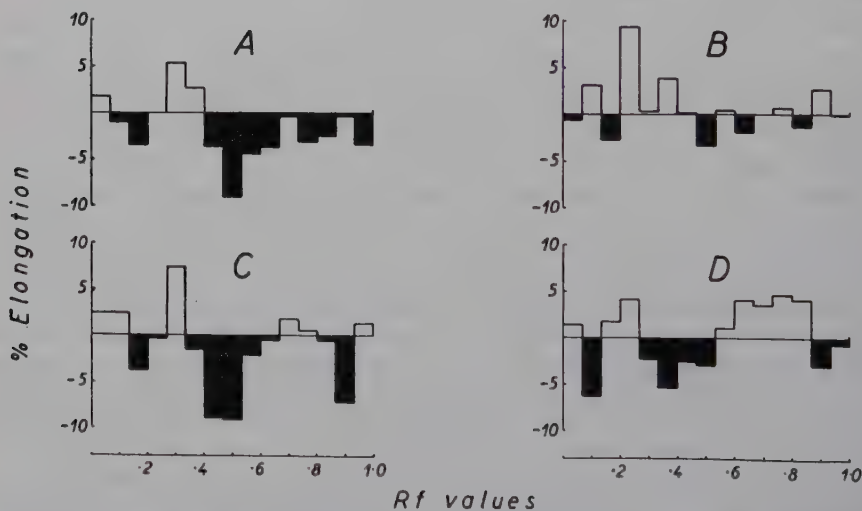


Figure 1. Biological determination of chromatograms of the acid fraction of ether extracts of potato peel from the variety *Eigenheimer*. A and C from extracts prepared on September 19 from unsprouted potatoes, B and D from extracts prepared on October 3 from sprouted potatoes (cf. the text). In A and B extracts from 5 g. potato peel on each chromatogram, in C and D extracts from 10 g. Abscissa: Position of the paper segment on the chromatogram in Rf units. Ordinate: Growth of wheat coleoptiles in per cent of the growth of the control segment.

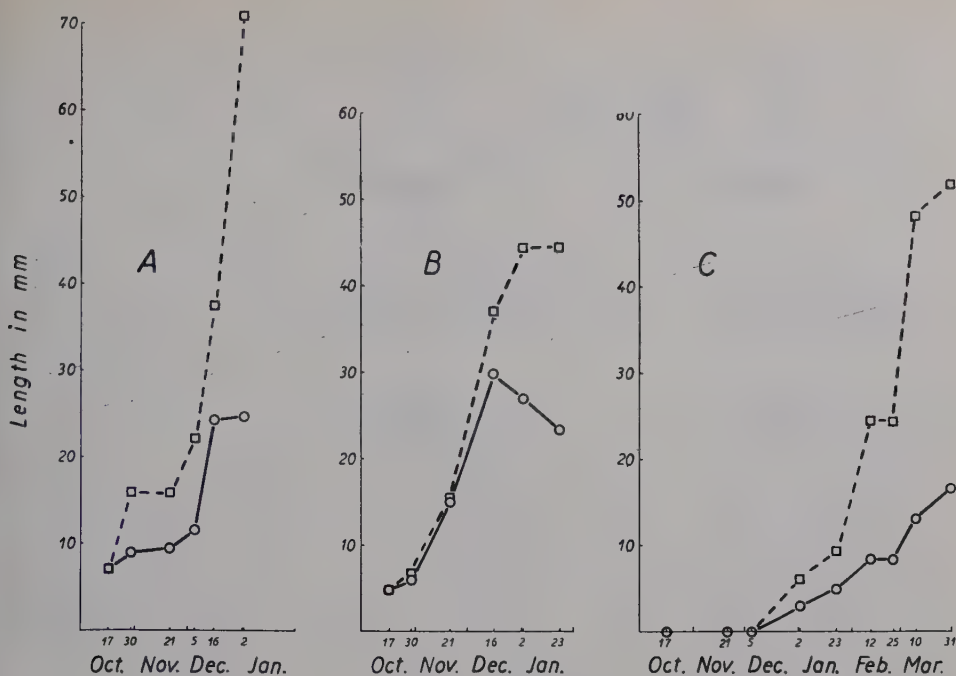


Figure 2. Sprouting experiments with potatoes of the varieties *Eigenheimer* (A), *Magnum bonum* (B) and *Majestic* (C). \circ = length of the longest sprout 14 days after the beginning of the experiment (average of 15 potatoes), \square = length at the same time of all at least 2 mm. long sprouts of one potato (average of 15 potatoes). See the text for the experimental conditions. Abscissa: Starting time of the different sprouting experiments. Ordinate: Length of sprouts in mm.

19, and in extracts of peel from sprouted potatoes, prepared on October 3, was determined. The results, which are seen in Figure 1, show clearly that the first extract contained considerably more acid inhibitor than the latter. The extracts differed in regard to the amount of the acid inhibitor which on chromatographing in isopropanol—ammonia—water had R_f values between 0.4 and 0.7, i.e., inhibitor β .

Later on during the fall and winter repeated experiments were carried out with *Eigenheimer*. Peel of this variety was extracted on October 17 and 30, November 21, December 5 and 16, and January 2. The results of the sprouting experiments carried out at the time of each extraction, which are reproduced in Figure 2 A, showed that even if the potato during October, November, and the beginning of December had a certain sprouting ability, it nevertheless exhibited a distinct inhibition of sprouting. This was strongest in potato planted on October 17, somewhat less in that planted on October 30

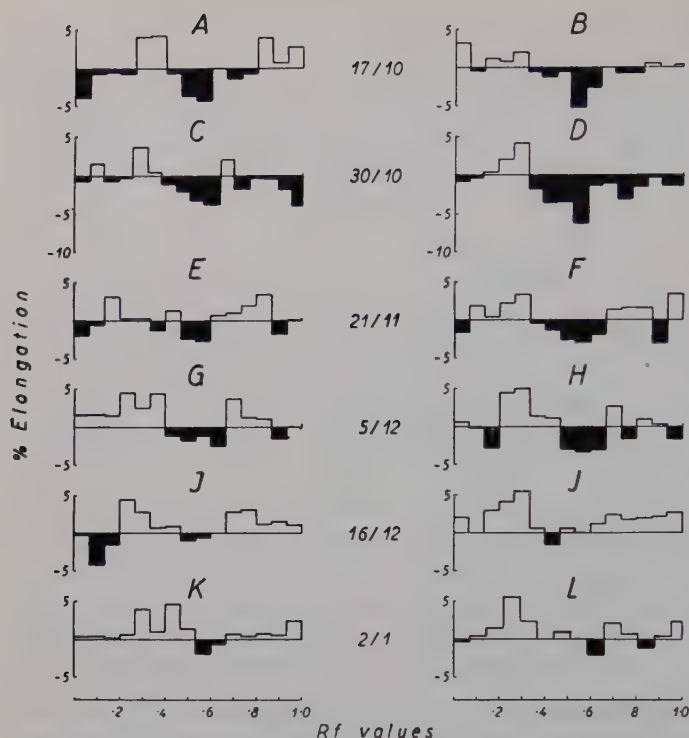


Figure 3. *Biological determination of chromatograms of the acid fraction of ether extracts of potato peel from the variety Eigenheimer prepared on different occasions. Two chromatograms are examined for each extract. Extract from 2.5 g. potato peel is transferred to each chromatogram. The dates given for the different experiments denote the time of the preparation of the extracts. Abscissa and ordinate as in Figure 1.*

and November 21, and still less pronounced in potato planted on December 5. The shoots of potato planted on December 16 and January 2 grew considerably more rapidly, and this potato had evidently left the rest period completely.

In comparing the results of this sprouting experiment with the histograms of the biologically tested chromatograms of the different extracts (Figure 3), it is found that when the inhibition of sprouting was strongest during October, the extracts' content of inhibitor β was high (Figures 3 A—D). The inhibitor content was somewhat less in the extracts from November and December 5 (Figure 3 E—H). In the extract from December 16 scarcely any inhibitor β could be demonstrated, as well as in the extract prepared on January 2 (Figure 3 I—L).

From the chromatograms it is evident that in addition to an acid auxin with the Rf value 0.2—0.4, obviously indole acetic acid, an auxin with high Rf value was present. The content of indole acetic acid was unchanged during the entire experimental period. The content of the other auxin possibly rose somewhat toward the end of the rest period. This does not necessarily

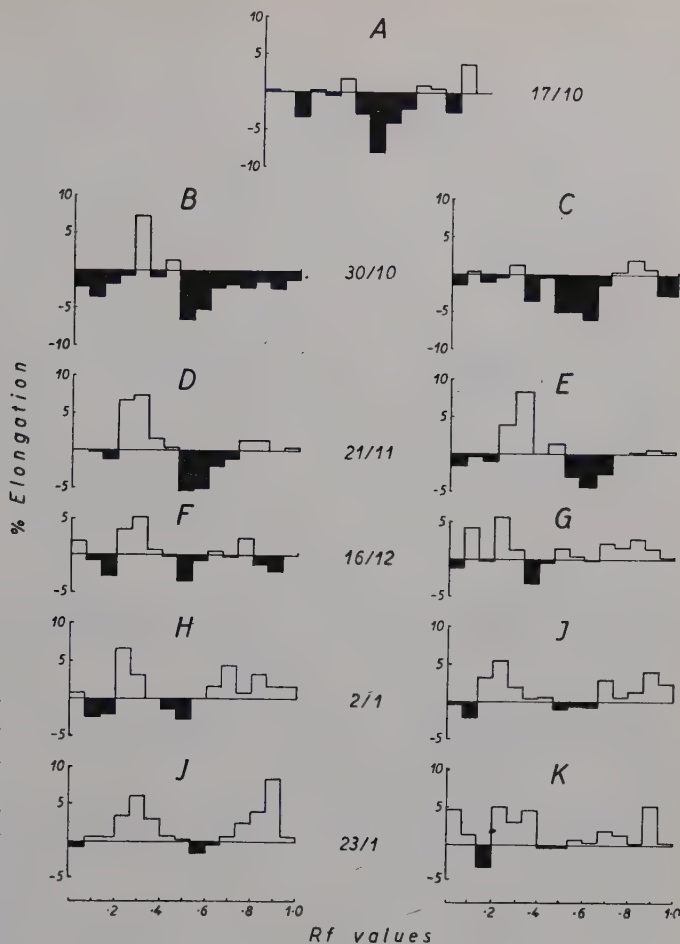


Figure 4. Biological determination of chromatograms of the acid fraction of ether extracts of potato peel from the variety *Magnum bonum* prepared on different occasions. Otherwise as in Figure 3.

mean, however, that this auxin is formed toward the end of the rest period, as Blommaert (1954) assumed, but the rise may have been apparent due to the fact that this substance has earlier been masked by the high content of inhibitor β . The increase in the content of this auxin is, moreover, exceedingly slight. Since the main interest has been concentrated on the content of inhibitors and not on the content of auxin, very small amounts of extract have been transferred to the chromatograms so that eventual auxins should not occupy such large parts of the chromatograms that they affected the determinations of the inhibitors. The auxin determinations are therefore rather uncertain.

Magnum bonum. This potato variety, which was harvested on October 2,

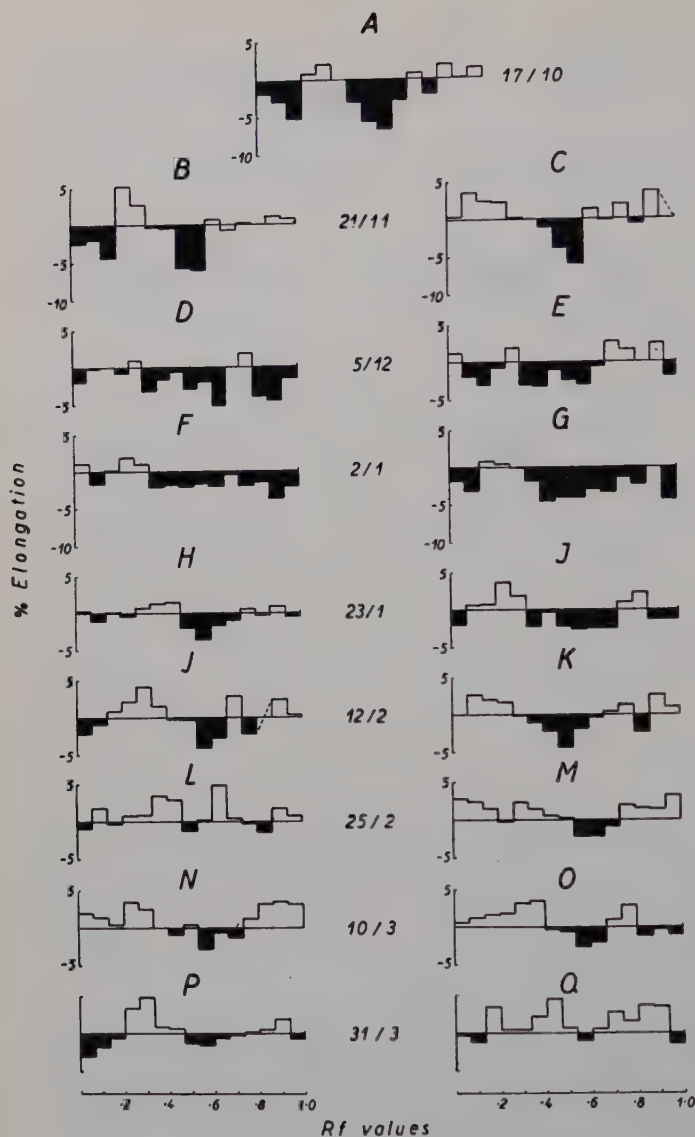


Figure 5. Biological determination of chromatograms of the acid fraction of ether extracts of potato peel from the variety *Majestic* prepared on different occasions. Otherwise as in Figure 3.

1957, was examined on October 17 and 30, November 21, December 16 and January 2 and 23. The sprouting experiments show that the inhibition of sprouting in this variety was strongest in October, less pronounced on November 21 and exceedingly slight in potato planted on December 16 or later (Figure 2 B). Regarding the inhibitor content of the extracts, there was an abundance of inhibitor β in the extracts that were prepared on the first two

occasions of extraction (Figure 4 A—C) and a somewhat smaller amount in the extract prepared on November 21 (Figure 4 D and E). Very little was found in the extract from December 16 (Figure 4 F—G). Practically no inhibitor β could be detected in extracts prepared at a later date (Figure 4 H—K).

Also in *Magnum bonum* no distinct differences could be demonstrated in the content of indole acetic acid between the extracts prepared on different occasions. On the other hand, in connection with the termination of the rest period or after this had taken place there was a slight increase in the content of an auxin with a high Rf value.

Majestic. This variety, which was harvested on September 14, 1957, was examined on October 17, November 21, December 5, January 2 and 23, February 12 and 25 and March 10 and 31. At the time for starting the first three sprouting experiments the potato was generally unable to sprout at all (Figure 2 C). Potato planted on January 2 and 23 had a slight sprouting ability. The sprouting ability was gradually increased in potato planted on February 12 and 25 and March 10 and 31.

The histograms of the biologically tested chromatograms of the different extracts show that considerable inhibitor β was present in the extracts which were prepared up to and including January 2 (Figure 5 A—G). The inhibitor content in the extracts that were prepared on January 23 and February 12 was somewhat lower (Figure 5 H—K). In extracts prepared on February 25 and March 10 and 31 there was even less inhibitor β (Figure 5 L—Q).

Also in *Majestic* the content of indole acetic acid during the entire experimental period appears to have been the same. It was, moreover, lower than in the other examined varieties. Also in this variety there is an indication of formation of an auxin with a high Rf value toward the end of the rest period.

Discussion

The reported experiments demonstrate that in all three of the examined potato varieties there is a distinct correlation between the occurrence of inhibitor β in the peel layer of the respective variety and the inhibition of sprouting in this variety. As soon as the inhibitor content of the peel layer has begun to decrease, there is also a decrease in the inhibition of sprouting. The degree of sprouting inhibition on the different experimental occasions gives information on how deep the rest has been on these occasions.

In *Eigenheimer* and *Magnum bonum*, whose rest periods were shorter than the rest period of *Majestic*, inhibitor β could no longer be demonstrated already in the middle of December. The same inhibitor occurred, on the other

hand, in a large amount in the peel layer of Majestic as late as the middle of February, but it disappeared later from the peel layer in connection with the increased sprouting ability.

From the histograms in Figures 3, 4 and 5 it is evident that inhibitor β in the extracts prepared on different occasions can have somewhat varying Rf values. The substance occurs at times only within a narrow limited part of the chromatograms, whereas on other occasions it spreads over larger parts of the same. This is probably due to the fact mentioned in the introduction that inhibitor β is composed of a complex of organic acids and that the composition of this complex can vary during different stages of the rest.

The experimental results lend further support to the assumption proposed by Hemberg (1952) that it is the acid inhibitors in the peel layer of the potato which regulate its rest. Results with the same indications have, as earlier reviewed, also been obtained by Blommaert (1954) and Varga and Ferenczy (1956 and 1957 a). These investigators, however, did not examine several potato varieties nor did they carry out any sprouting experiments, except that Varga and Ferenczy (1957 a) stored their potato at 20°C and examined it also after it had begun to sprout.

That the rest can be correlated with the occurrence of inhibitor β in the resting organ has been demonstrated also for tree buds. Blommaert (1955) found that resting buds of peach contained an abundance of an acid inhibitor, which judging from the chromatographic data was inhibitor β . This disappeared from the buds in connection with the breaking of the rest. Hemberg (1958 b) showed that resting terminal buds of ash contained a similar inhibitor, whereas buds whose rest during the course of the winter was totally or partly broken were practically free from the inhibitor in question.

Summary

The acid fraction of ether extracts prepared on different occasions from the peel of three different potato varieties with unequally long rest periods, Eigenheimer, Magnum bonum, and Majestic, were examined with the help of paper chromatography for their content of inhibitor β . At the time of each extraction the sprouting inhibition (rest) of the different varieties was also determined. In all three varieties there proved to be a distinct correlation between the content of inhibitor β at the time of the different extractions and the sprouting inhibition. The experiments thus lend further support to the assumption that the rest of the potato is regulated by the occurrence of acid inhibitors, i.e., inhibitor β , in the peel layer of the potato.

This investigation has been partly supported by contributions from the Swedish Science Research Council. The author wishes to thank Fil. dr. B. Emilsson, Nynäshamn, for his willingness to permit the cultivation and storage of the potatoes used in the investigation. The wheat employed in the test method has been supplied by A.B. Weibull, Landskrona, through the courtesy of Fil. dr. O. Gelin. The author is also indebted to Mrs. Maija-Liisa Holmberg and Mrs. Janina Pisarska for their valuable technical assistance in the performance of the experiments.

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Simple Devices for Aseptic Culture of Seed Plants

By

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(Received May 5, 1958)

Where no special room is available for aseptic transfer of seeds, seedlings and tissues, some device is necessary to enable such transfers to be made without exposing the culture objects to contamination by air. The flasks described below have proved to meet the case.

a. S-tube flasks

Figure 1 represents a model particularly applicable to water plants but also to many others which can be grown wholly or partly submerged. Pyrex Erlenmeyer flasks are provided with sigmoid side tubes for sterilization and aseptic transfer. In the present case $\frac{3}{4}$ litre flasks were employed, the side tube having an inner diameter of 10 or 11 millimetres. For autoclaving, the mouth of the flask is covered with aluminum foil and paper parchment, and the side tube is closed with a rubber stopper. It is advisable to provide the flask neck with a cotton wool girdle before covering and to fasten the cover with a rubber ring. Talc can be used to prevent the rubber stopper from becoming stuck in the glass tube. When seeds are to be sterilized, a suitable amount of a 10 % hydrogen peroxide solution is poured into the side tube, after which the seeds are put in. In many cases about half an hour will suffice to rid the seeds of external contamination; as is well known, internal contamination cannot be got rid of in this way. To facilitate wetting of the seeds by the hydrogen peroxide solution, these can be pretreated with alcohol

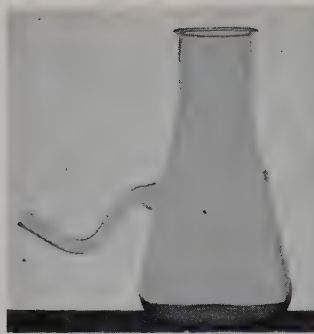


Fig. 1.

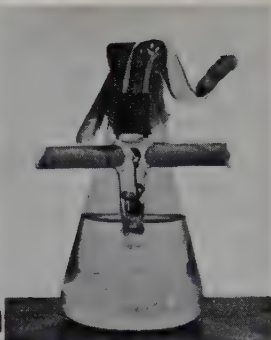


Fig. 2.

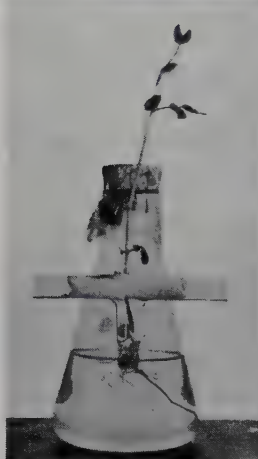


Fig. 3.



Fig. 4.

Figure 1. *S-tube flask*. Height 21 cm.

Figure 2. *Crosstube flask with the sigmoid sterilizing tube and a pea seedling*. Height 18.5 cm.

Figure 3. *Crosstube flask with a more advanced pea seedling, without the sterilizing tube*.

Figure 4. *The same flask as in Figure 3, but in side view*.

or detergents. When sterilization is completed the hydrogen peroxide solution is allowed to flow out by loosening the rubber stopper slightly, and the seeds are rinsed with the sterile nutrient solution by inclining the flask in a suitable manner. Care should be taken not to allow the hydrogen peroxide solution to enter into the nutrient solution, and to prevent contamination of the nutrient solution by air bubbles passing through the side tube. When the $\frac{3}{4}$ litre flasks are supplied with 250 millilitres of nutrient solution, the above-mentioned manipulations can be performed without difficulty.

Aseptic seedlings or explants, if small enough, can be introduced into the flask in a similar way. For this purpose the flask must be inclined so that the

side tube becomes filled with the sterile nutrient solution. Then the rubber stopper is opened cautiously, the culture objects are put in and rinsed repeatedly, the rubber stopper slightly loosened, so as to allow nutrient solution to flow out. Finally the rubber stopper is closed tightly and the culture objects are rinsed into the flask. After this the side tube should be rinsed with hydrogen peroxide solution, which must not be allowed to enter into the nutrient solution. Most of the transfers made in this way have been successful.

b. Crosstube flasks

Figures 2—4 represent flasks which are intended for plants forming aerial shoots. An Erlenmeyer flask is provided with a crosstube into which the seed is conducted when it has been sterilized in the sigmoid tube attached to the crosstube by a piece of rubber tubing. For autoclaving the horizontal arms of the crosstube and the sigmoid tube must be closed with cotton wool plugs, and the mouth of the flask as in the preceding case. In the horizontal arms the cotton wool plugs should be loose at the proximal ends, tighter at the distal ends. Between the two plugs a free space must be left for the shoot to grow through, after which the two plugs are pressed tightly together and the sigmoid tube is removed (Figure 3). For sterilizing, an appropriate amount of a 10 % hydrogen peroxide solution is poured into the sigmoid tube, the seed is put in, and the orifice closed with cotton wool. After about half an hour, the hydrogen peroxide solution is allowed to flow out through the cotton wool plug, which is not removed. Drainage is facilitated if dry cotton wool is pressed against the wet plug. During this procedure the sigmoid tube must be turned towards the flask. In order to conduct the seed into the crosstube, the sigmoid tube is turned in the opposite direction and tapped so that the seed drops into the crosstube, in which it will be supported on the basal constriction made by four indentations. In this position the seed should remain in contact with the nutrient solution. The root will then grow through the constriction into the flask, and the shoot upwards into the sigmoid tube. If the stem takes a central position in the crosstube it can readily be isolated by cotton wool plugs put in the horizontal arms, but sometimes the stem may adhere to the glass tube, making effective isolation more difficult. The cotton wool plugs must not be allowed to absorb nutrient solution. To avoid this, it is advisable to place the flasks in a tilted position in the autoclave.

The crosstube flasks have been adapted for culturing peas and beans. For these plants the crosstube calibre can be 12 millimetres, and the flask may have a volume of half a litre. When supplied with 400 millilitres of nutrient

solution, pea plants may be grown for two or three months, within which time they may form flowers and pods. In the long run too much water will be lost by transpiration. For short-time experiments 250 millilitre flasks have been used.

Summary

Two devices have been described for the aseptic culture of submerged and aerial higher plants.

Production of High Laccase Yields in Cultures of Fungi

By

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Although the phenol-oxidizing enzyme, laccase, was first discovered in the latex of the lacquer-trees, it was soon found also in other materials, for instance in the fruit-bodies of many basidiomycetes (Bertrand and Bourquelot 1895). Of considerable interest is the regular occurrence of this oxidase in the lignin-decomposing fungi. Following Bavendamm's observation (1928) that fungi belonging to this physiological group oxidized phenolic compounds, the oxidizing agent was identified as laccase (Fåhraeus 1949, Lindeberg and Fåhraeus 1952, Higuchi 1953, Lyr 1958).

The first successful attempts to purify the lacquer tree laccase were made by Keilin and Mann (1939, 1940). Later D. Bertrand (1944) and Tissières (1949) described preparations from the same source with somewhat higher specific activity. Using the wild mushrooms, *Lactarius piperatus* and *Russula foetens*, respectively, Yakushiji (1937) and Gregg and Miller (1941) reported the partial purification of laccase. Quite recently, the cultivated mushroom (*Psalliota*) has also been used for this purpose (Freudenberg *et al.* 1958). However, due to the very complex nature of this material, the enzyme purification requires a large number of successive steps, and heavy losses are inevitable. Nevertheless, such fruit-bodies are still considered the best source of tyrosinase (Kertész and Zito 1957).

As an alternative source of laccase, pure cultures of lignin-decomposing fungi may deserve attention. Our results indicate that some *Polyporus* species if cultured on special nutrient media are a suitable starting material for the production and purification of laccase. Two circumstances, in particular,

facilitate the preparation from this source. First, the laccase is almost quantitatively secreted into the culture medium. Second, the enzyme is largely inducible, which means that the addition of certain substances results in a greatly increased production of laccase, whereas the formation of other enzymes is not influenced. Due to this fact, the laccase becomes the major constituent of the secreted protein (Malmström, Fåhraeus and Mosbach 1958).

The aim of the present work was to ascertain the optimal culture conditions for production of laccase in amounts sufficient for preparative work. Particularly, the influence of following factors was investigated a) the type of organism and b) the nutritional factors including enzyme induction by various substances.

Materials and Methods

In the experiments recorded here, dicaryotic mycelia of the following fungi were used: *Marasmius graminum* Berk. & B., *M. scorodionius* Fr., *Stereum hirsutum* Fr. (2 strains), *S. purpureum* Fr., *Polyporus abietinus* Fr., *P. hirsutus* Fr., *P. zonatus* Fr. (2 strains), and *P. versicolor* Fr. (9 strains).¹ Some of these have been used in earlier work (7) while some, among them 7 strains of *P. versicolor*, were isolated later. Mycelia were obtained from diluted basidiospore suspensions (*P. abietinus*, the *Stereum* species), or by aseptic preparation from fruit bodies. For some strains of *P. versicolor*, a disinfection procedure with α -naphthol (7) proved helpful.

All strains of *P. versicolor* originated from specimens collected on beech or oak stumps at Uppsala, Sweden (no. 1) or Hillerød, Denmark (no. 11—14) except one (761), which was obtained through the courtesy of Dr. E. Hellmers, Copenhagen.

The substrates used in the present work were malt and yeast extracts or certain chemically defined media described in earlier publications: Bf-2 and some minor modifications thereof (13). As culture vessels 125 ml. Erlenmeyer flasks containing 20 ml. of medium were used for surface cultures, and one liter Erlenmeyer flasks containing 500 ml. of medium placed on a rotary shaker for submerged cultures. Inoculation of the experiments was usually made by adding a mycelium suspension (13).

The following chemicals were used in a number of experiments reported below: guaiacol c.p. Merck, indole-3-acetic acid c.p. Merck, *o*-, *m*-, and *p*-toluidine hydrochloride c.p. British Drug House, 2,3- and 3,4-xylydine c.p. Fluka, 2,4-, 2,5-, 2,6-, and 3,5-xylydine c.p. Eastman. These substances were ordinarily used without further purification; in a few experiments with 2,5-xylydine, the substance was purified by distillation in vacuo before use. The substances were as a rule dissolved in 50 % alcohol before addition.

Oxidase determinations were made colorimetrically or by the Warburg manometric technique. For colorimetric measurements, an E.E.L. portable colorimeter with the blue filter No. 303 was used, the reaction mixture containing 1 ml. 0.1 *M* catechol,

¹ In the literature, the four last-mentioned species are sometimes referred to the genera *Coriolus*, *Polystictus*, or *Trametes*.

0.1—1.0 ml. test solution, 5 ml. 0.1 *M* acetate buffer pH 5.0 and water to a final volume of 11 ml. The light extinction was measured after 10 minutes.

Manometric determinations were carried out as earlier described with catechol as a substrate (13).

Laccase Activity of Different Species and Strains of Basidiomycetes

Both the tyrosinase type and the laccase type of phenoloxidases occur in the basidiomycetes. Tyrosinase has been found particularly in the fleshy fruit-bodies of these fungi, but it occurs also in the mycelium of certain species when grown in ordinary culture media (Boidin 1951, Lindeberg and Holm 1952, Lyr 1956, Piroard 1956). The laccase type, however, seems to be more common under such circumstances: Boidin found laccase in 102 species and tyrosinase in 55; similar proportions were given by Piroard. Lyr mentions that, among 182 species, 75 were able to form laccase, while 62 formed tyrosinase.

The number of potential sources for laccase production thus seems to be fairly large. Unfortunately, most basidiomycetes are extremely slow-growing which makes them unsuitable objects for preparative enzyme work. There are, however, a few groups of basidiomycetes, particularly among the wood-inhabiting species, which show vigorous growth on suitable laboratory media and, therefore, offer better prospects in this regard.

In previous work, good results have been obtained with a strain of *Polyporus versicolor* (strain no. 1). It seemed worthwhile to test also other fungi which gave a strong positive response on agar media containing phenols according to Bavendamm (1928). In the following paragraphs, results obtained with a number of *Polyporus*, *Stereum*, and *Marasmius* species are recorded.

In the first experiment, the test fungi were cultured on malt extract medium. Analyses of mycelial weights and oxidase activity of the filtrates were made after 7, 10, 14, 21, and, in some instances, 28 days. The results are illustrated by Figure 1.

It is evident from the Figure that large differences in oxidase activity exist between the different fungi. Thus, the three *Stereum* strains had a very low activity as compared with most *Polyporus* strains. The shape of the oxidase curves is, however, similar in all cases. Evidently, high oxidase activities do not always correspond to high mycelial weights. For instance, *P. zonatus* 1 with a moderate growth in malt extract medium has a very high oxidase activity, while *P. zonatus* 2 with a much larger mycelium production shows slightly lower oxidase activity.

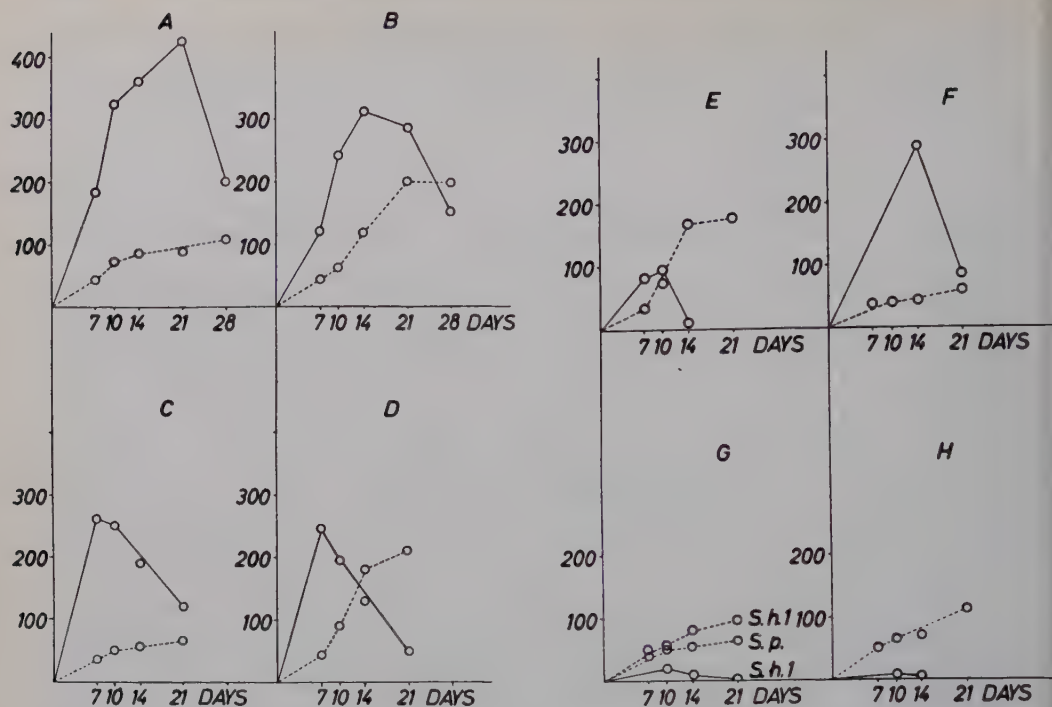
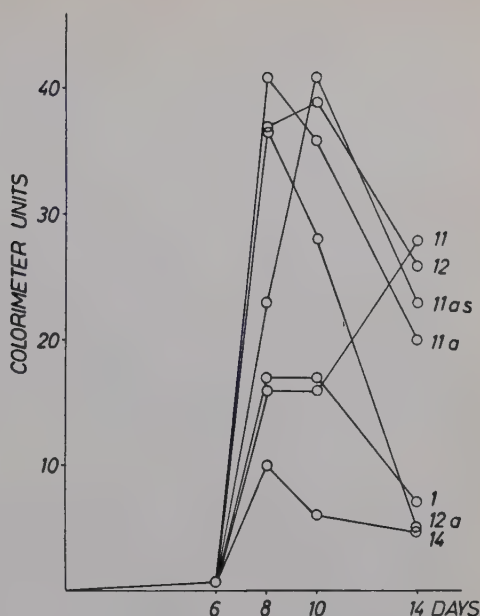


Figure 1. Growth and laccase production in cultures of various *Polyporus* and *Stereum* species. ---○--- mycelial dry weight in mg per flask; —○— laccase activity of the medium in $\mu\text{l. O}_2$ per hour per ml. culture liquid. A=*P. zonatus* 1, B=*P. zonarius* 2, C=*P. abietinus*, D=*P. versicolor* 1, E=*P. hirsutus* 1, F=*P. hirsutus* 2, G=*S. hirsutum* 1, *S. purpureum*, H=*S. hirsutum* 2.

Since previous work with *P. versicolor* 1 had shown that certain substances induced a strong oxidase formation (9—13), it was thought that fungi with a weak oxidase activity on ordinary media might give other results in the presence of special inducing substances. Therefore, an experiment was made with some such compounds added to cultures of *Stereum hirsutum*, *Marasmius graminum*, and *M. scorodoni* (Table 1). The results show, however, that the oxidase activity was generally low, although good growth had occurred. The addition of compounds which are very powerful inducers in cultures of *Polyporus versicolor* had increased the activity only to a small degree. In the *Marasmius* species, a certain non-induced enzyme formation can be observed. In *Stereum hirsutum*, only guaiacol was effective as an inducer.

From the preceding results, the most promising fungi for further study seemed to be some *Polyporus* species. Since large differences between strains

Figure 2. Laccase production of different strains of *Polyporus versicolor* in the synthetic medium Bf-2. *o*-Toluidine hydrochloride (10^{-3} M) added after 6 days growth. Laccase activity expressed as colorimeter units (0.5 ml. enzyme, total volume 11 ml.). The results for strain 13 and 761 which are almost identical with those for 1 and 14, respectively, are not plotted.



were apparent (Fig. 1), a number of isolates of *P. versicolor* were now compared. A preliminary experiment with some of these conducted shortly upon their isolation (in 1952) had established a good activity, although variation between strains was great.

Nine strains of *P. versicolor* were tested on two substrates: Bf-2 and Bf-2+2 % yeast extract. To both media, *o*-toluidine was added as an inducer. The toluidine was added at the start of the yeast extract series, but not until after 6 days in medium Bf-2 because of the toxicity of the inducer in the synthetic medium. Duplicate flasks

Table 1. Effect of *o*-toluidine, indole-3-acetic acid (IAA), and guaiacol on the growth and laccase production of *Stereum hirsutum* '1, *Marasmius graminum*, and *M. scrodonius*. Medium Bf-2, substances added after 8 days growth (*S. h.* and *M. g.*) or at the start (*M. sc.*).

Harvest after 13 and 30 days, respectively. 6 replicate flasks.

Substance added M	Mycelial dry weight in mg.			Laccase activity, μ l. O_2 /hr. per culture		
	<i>St. h.</i>	<i>M. g.</i>	<i>M. sc.</i>	<i>St. h.</i>	<i>M. g.</i>	<i>M. sc.</i>
None	164	255	141	50	650	990
<i>o</i> -Toluidine 10^{-4}	167	259	146	50	460	1700
" 10^{-3}	159	223	—	50	1000	—
IAA 10^{-4}	137	250	148	50	510	1400
" 10^{-3}	121	236	3.5	50	970	100
Guaiacol 10^{-4}	162	262	—	70	490	—
" 10^{-3}	120	244	—	410	870	—

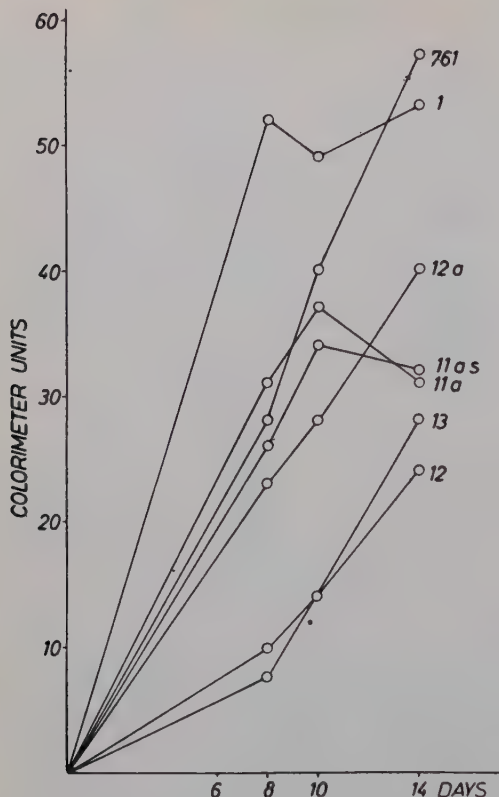


Figure 3. Laccase production of different strains of *Polyporus versicolor* in medium Bf-2 + 2% yeast extract (Difco). *o*-Toluidine hydrochloride (10^{-3} M) added at time 0. Laccase activity expressed as in Figure 2. The results for strain 11 and 14 which are almost identical with those for 11 a and 761, respectively, are not plotted.

were taken out for analysis after 8, 10, and 14 days incubation. The oxidase activities of the solutions were measured colorimetrically.

The results are shown in Figures 2 and 3 and Table 2.

From the Figures it is clearly seen that the formation of laccase in the synthetic medium is insignificant until the inducer is added. Then a sharp rise in activity can be observed, but the maximum values vary considerably between strains. In most cases, the maximum is practically attained as soon as after 2 days (6 strains). 2 strains had reached the maximum after 4 days, and only in one case maximum production was somewhat delayed. Thereafter, a rapid decline occurs, particularly in the most active strains (11 a, 11 as, 12, 12 a).

The mycelial weights, given in Table 2, show that no positive correlation between growth and enzyme production exists. For instance, the strong oxidase producer, strain 12, shows a rather low mycelium production after 10 days as compared with most other strains.

Table 2. *Growth of 9 strains of Polyporus versicolor on medium Bf-2, and Bf-2+yeast extract. o-Toluidine added as an inducer. Duplicate flasks. Results expressed as mycelial dry weight in mg per flask.*

Strain	Medium Bf—2			Medium Bf—2 + yeast		
	8	days 10	14	8	days 10	14
1	116, 122	140, 147	182, 175	204, 208	204, 198	163, 163
11	55, 49	151, 137	215, 228	195, 195	175, 162	145, 148
11 a	91, 94	160, 167	229, —	202, 189	191, 167	148, 148
11 as	45, 68	151, 136	205, 180	194, 195	184, 185	149, 147
12	39, 44	90, 83	207, 215	185, 179	191, 180	145, 144
12 a	113, 105	173, 171	187, 182	202, 203	181, 190	113, 110
13	20, 32	68, 70	166, 156	189, 171	191, 193	157, 153
14	152, 166	151, 158	172, 164	188, 188	161, 160	122, 121
761	118, 115	192, 163	136, 149	137, 120	139, 120	73, 71

In the yeast extract series, a considerable non-induced formation of oxidase takes place. This fact has been discussed earlier (10). As a rule, however, *o*-toluidine increased the oxidase production also in this medium. In contrast with the synthetic medium with toluidine, no decrease in activity was found after 14 days, in most cases even a further increase. This is probably due to the pronounced autolysis of the mycelium which is apparent from the mycelial weights given in Table 2. This phenomenon was also noted with the synthetic A medium in previous work (8).

From the data presented, it would seem that most of the strains used in this experiment have a good laccase-producing capacity, but that optimum yield is not obtained for all strains in the same medium. This is most obvious for the strains 14 and 761, which gave a very good activity in the yeast extract medium but much less so when cultivated in the Bf medium. Strain 12 shows the opposite behaviour.

On the basis of these and other experimental results, strain no. 11 a was chosen for subsequent experiments on a large scale for production of laccase. Some experiments recorded in the following sections were, however, made at an earlier date, and *P. versicolor* strain 1 was then ordinarily used.

Influence of Various Culture Conditions

a. Effect of submerged culture

Dion (1952), Tonhazy and Pelczar (1954), and Boulter and Burges (1955) have used the submerged culture method successfully in culturing *Polyporus versicolor*. Also in the present work, rapid growth was obtained with this

method. The laccase activity of such cultures compared favourably with that obtained in surface cultures with the same medium. Since larger volumes per flask could be chosen, this culture method is obviously preferable for enzyme production.

b. Effect of synthetic media and addition of inducing compounds

Earlier results have shown that high laccase activities may be obtained in several types of media, *viz.*:

- 1) malt or yeast extract solutions (8, 10),
- 2) synthetic media with inducing substances added (12, 9, 10),
- 3) media without any exogenous inducer following autolysis of the mycelium (8).

For the production of laccase for purification it is clearly desirable to choose a medium where a high ratio of laccase to other enzyme protein is obtained. Since media containing yeast extract or similar supplements will stimulate the formation of several different enzymes, such solutions would seem less suitable for this purpose, even if the laccase formation is strong. The same is true for solutions in which autolytic processes are going on. Since *Polyporus versicolor* showed vigorous growth and laccase formation on certain chemically defined media, such media have been preferred. Actually, some experiments with yeast extract media showed that the resulting crude enzyme solution was highly coloured and contained more inactive protein than the corresponding solution obtained by using a synthetic medium.

The choice of a suitable inducer becomes highly important, when synthetic media are used. A great number of different organic substances have been compared in this respect, some of the results have been published earlier (9—13). Among the most promising compounds were the toluidines (10) and a xyldine (11). Some other very powerful inducers were often more toxic in effective concentrations (chlorophenols, 8-hydroxyquinoline, indole-3-acetic acid).

Two further experiments, in which the different isomers of toluidine and xyldine were tested, will be reported here.

In the first experiment, *P. versicolor* 1 was tested in 125 ml. flasks in surface culture with the inducer (10^{-4} M) added after 6 days growth and analysis made after 10 days. In the second one, strain 11 a was tested in one liter flasks incubated on the shaker. The concentration of inducer was in this case 2×10^{-4} M. Addition was made after 4 days and the flasks were analyzed after 7 days.

The results of these experiments are given in Table 3.

Although the conditions of the two experiments were very different, 2,5-xyldine proved to be the most efficient inducer in both cases. Some vari-

Table 3. *Effect of toluidines and xylydines on growth and laccase formation of 2 strains of Polyporus versicolor* (1 and 11 a). *P. versicolor* 1 cultured in 125 ml. flasks, inducer (10^{-4} M) added after 6 days, analyses made after 10 days, 6 replicate flasks. *P. versicolor* 11 a cultured in one liter flasks on the shaker, inducer (2×10^{-4} M) added after 4 days, analyses after 7 days. Duplicate flasks. Medium Bf-2 with 25 μ g. Cu/liter (1) or 250 μ g. Cu/liter (11 a).

Substance added	Strain 1		Strain 11 a	
	Mycelial dry weight in mg.	Laccase activity μ l. O_2 /ml. hr.	Mycelial dry weight in mg.	Laccase activity μ l. O_2 /ml. hr.
None (at the time of addition)	70 \pm 0.95	—	785	—
None (at harvest) ...	171 \pm 2.7	2	1435, 1484	97
o-toluidine	169 \pm 1.1	48	1724, 1761	1030
m-toluidine	168 \pm 0.71	75	1840, 1890	1465
p-toluidine	166 \pm 0.85	27	1400, 1405	1055
2, 3-xylydine	174 \pm 1.1	17	1503, 1593	260
2, 4-xylydine	175 \pm 0.98	41	969, 1103	124
2, 5-xylydine	174 \pm 2.5	161	2242, 2202	1880 ¹
2, 6-xylydine	166 \pm 1.7	86	849, 818	74
3, 4-xylydine	176 \pm 3.0	98	1982, 1943	1235
3, 5-xylydine	173 \pm 2.3	114	2005, 2009	1280

¹ Due to the presence of an inhibitor, this value is certainly too low.

ation is noted regarding the other substances. In the second experiment, two of the xylydines inhibited growth severely, which may explain the very low laccase yield in these cases. On the other hand, several of the added substances greatly stimulated growth. This is further discussed on p. 640.

The optimum concentration of 2,5-xylydine has been tested in several experiments, using the range 10^{-4} to 10^{-3} M. Results are not yet definite, but it seems that a concentration of 2×10^{-4} M is preferable because higher concentrations, although sometimes giving larger enzyme activities, also give rise to an increased formation of polysaccharide.

c. Age of the culture

From the type of curves obtained in many experiments (see Figures 1 and 2) it is seen that the period of high enzyme activity in the nutrient solution is often rather restricted. This means that the time of addition of the inducer on one hand, and the time of harvest on the other, is important for preparative work. An early addition of the inducer may retard mycelial growth, and too late an addition probably stops enzyme production at a low level owing to exhaustion of necessary energy-yielding or building materials. Table 4 gives the results from a typical experiment. In this, the highest activity was obtained when the inducer had been added after 3 or 4 days growth. Maxi-

Table 4. Effect of age of the culture at the addition of inducer (2×10^{-4} M 2,5-xylydine) on the laccase production in *Polyporus versicolor* 11 a. Medium Bf-2. Results given as colorimeter units. (0.1 ml. enzyme, total vol. 11 ml.)

Xylydine added after days	Activity measured after days		
	6	7	8
0	11	7	5
2	19	20	22
3	32	22	23
4	31	23	24
5	15	12	11
6	2	9	9

imum was reached within 6 days after inoculation. On the basis of these results, the inducer was usually added after 3 or 4 days growth, and the solutions were collected at the time of maximum activity (after an additional 2 or 3 days). After separation of the liquid medium, the mycelia may be used for production of a second batch of enzyme by adding fresh nutrient medium supplied with xylydine.

d. Copper content of the medium

Apart from special inducing substances, the composition of the basal medium influences the production of laccase. So far, we have only studied the effect of copper, which, as a constituent of the enzyme molecule, might be expected to affect the laccase formation. The medium used in most previous experiments (Bf or Bf-2) contains only 25 μ g. Cu per liter. The results from an experiment covering the range 25—10,000 μ g. Cu are presented in Table 5.

It is evident from Table 5 that copper has a great influence both on laccase and mycelium production, with a maximum at about 250 μ g. Cu per liter. Higher copper concentrations tend to inhibit growth, but laccase production remains rather constant up to the highest Cu concentration tested. In the series without xylydine, the growth was not significantly stimulated by increased copper concentrations and the laccase activity remained consistently low, amounting only to a few per cent of that obtained on addition of xylydine.

The strongly stimulatory effect of xylydine on growth, especially in the presence of copper, is similar to that observed in many earlier experiments (see Table 3) with various aromatic compounds. This remarkable effect deserves further study. An explanation could possibly be sought in a metal-

Table 5. *Effect of copper content of the medium on the laccase production of Polyporus versicolor 11 a.* Submerged culture, basal medium Bf-2. 2,5-xylidine (2×10^{-4} M, redistilled) added after 4 days growth, harvest after 7 days. Mycelial weight at the addition of xyloidine ca. 650 mg./flask. 3 replicate flasks.

Copper concentration µg. per liter	Mycelial dry weight in mg.	Laccase activity		
		Colorimeter readings (0.1 ml.)		Manometer readings (1.0 ml. µl. O ₂ /hr.)
		6 days	7 days	7 days
0	1915, 1851, 1863	6	4.5	650
25	1937, 1867, 1936	13	9	1010
25 (no xyloidine)	1524, 1673, 1748	—	0.7	90
100	2017, 2133, 1938	33	25	3030
250	2277, 2282, 2295	30	35	3450
250 (no xyloidine)	1616, 1548, 1698	—	0.8	97
1000	2004, 2020, 1905	25	33	3720
2500	1757, 1913, 2122	25	32	3240
2500 (no xyloidine)	1749, 1607, 1669	—	1.0	129
10000	1633, 1597, 1593	24	31	3900

chelating potential of some of these substances. However, preliminary experiments with 2,5-xyloidine and copper gave no indication of such a mechanism.

Concluding Remarks

The high yields of laccase obtained in pure cultures of *Polyporus versicolor* have opened the possibility of using such cultures as a starting material for purification of the laccase. A preliminary account of a suggested procedure is given by Malmström, Fåhræus and Mosbach (1958). Some of the most important steps in the production of active enzyme solutions are as follows.

P. versicolor is cultured on a rotary shaker in a chemically defined medium containing glucose, asparagine, mineral salts, and thiamin (Bf-2, ref. 13). The copper concentration should be at least 250 µg. Cu per liter. After some days growth, a compound inducing laccase formation is added. The most efficient inducer found so far is 2,5-xyloidine in a concentration of about 2×10^{-4} Mol/liter. After an additional 2 or 3 days the mycelia are filtered off, the filtrates being combined and the laccase precipitated in the cold by adding solid ammonium sulfate to saturation. After separation of the precipitate, dialysis and filtration, a concentrated enzyme solution is obtained which usually has a strong green colour. Its activity is somewhat variable; Q_{O₂}-values around 20.000 are frequently found (µl. O₂/hr. mg dry substance, 25°C, catechol at pH 5). Judging from the calculated value of about 40.000 for pure laccase (17, 24) the enzyme should already at this stage be about 50 % pure. Usually some 50—70 % of the laccase content of the nutrient medium is recovered by this procedure.

Details regarding the further purification of this crude enzyme solution by zone electrophoresis are given in the above-mentioned publication (24).

Summary

A number of basidiomycetes were cultured on liquid media and tested for laccase activity. Among these, some closely related *Polyporus* species (*Polystictus* group) appeared to be the most promising material for production of high yields of laccase. Some *Stereum* and *Marasmius* species, although showing fairly good growth and giving strong reactions on Bavendamm's media, produced only small amounts of laccase as determined by quantitative methods.

Nine strains of *Polyporus versicolor* showed great variation in laccase production on different media. Two strains (nos. 1 and 11a) were studied in more detail under varying culture conditions. In chemically defined media, the addition of special inducing compounds was necessary to obtain a high yield of laccase. The most efficient substance was 2,5-xylydine which also greatly stimulated growth. The age of the culture when adding the inducer and collecting the enzyme-containing solutions was also important. The copper content of the medium must be kept sufficiently high to permit maximum production of laccase.

On the basis of the results summarized above, a procedure has been adopted for the production of larger amounts of laccase from *Polyporus versicolor* for enzyme purification. The crude enzyme solutions obtained by a simple ammonium sulfate precipitation of the culture media and subsequent dialysis, normally had Q_{O_2} -values of around 20.000.

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Studies on the Interaction of Antiauxin and Native Auxin in Wheat Roots

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Introduction

The effect of the substance *p*-chlorophenoxyisobutyric acid (PCIB) on growth was first studied in wheat roots by Burström (6). PCIB at concentrations 10^{-6} — 10^{-5} *M* increase the root length, due to an increase of the cell length. Thus the effect of PCIB is opposite to that of supplied indole-3-acetic acid (IAA). Furthermore, the effects on root cell elongation by combinations of PCIB and IAA lend support to the view that the former substance acts as an antiauxin, probably by antagonizing the native auxin. This antagonizing action was proposed to be a simple blocking of IAA from its site of growth action, *i.e.*, IAA and PCIB have opposite actions (5, 6) on the two phases in cell elongation in the sense of Burström (4). PCIB was also found to be an ideal antiauxin for roots, devoid of any harmful effects in itself.

In a recent work (20) Libbert arrives at about the same results when studying interaction in wheat roots. He concludes that IAA exerts its action on elongation at two growth centres, and that PCIB competes with IAA at both these centres.

McRae and Bonner (21) treated *Avena* section elongation data by the methods of enzyme kinetics and concluded that PCIB is a true antiauxin and acts by competing with auxin for auxin-receptive sites within the plant. Fransson and Ingestad (9) showed that in *Avena* coleoptiles PCIB interacts also with the native IAA.

It should be kept in mind that one *isobutyric acid*, indole-3-*isobutyric acid*, has been reported to give stimulation of elongation of *Avena* coleoptile sections and wheat roots (8, 13). This compound has both auxin and anti-auxin properties, suggesting that PCIB too has both these properties. However, there is no indication at present of such an auxin-like character of PCIB.

If a competitive interaction takes place between IAA and PCIB according to the scheme outlined above, native IAA will be displaced from its natural active sites by externally added PCIB. Consequently it is very likely that such an addition results in a higher concentration of the free IAA. However it is to be expected that the increase in concentration will be rather small. It is the purpose of the present paper to investigate whether or not a measurable change in IAA concentration occurs in roots.

Methods

Cultivation of wheat plants. Soaked kernels of Fylgia spring wheat (obtained from Svalöv, Sweden) were spread over stainless wire screens. Each screen was placed over a glass-case, 15 cm. deep and containing ten litres of a nutrient solution. The screen was placed at half a centimetre above the surface of the solution. The wheat kernels were covered with moist filter paper and left to germinate. After two days the roots were just dipping into the solution, and the filter papers were removed. From then on the nutrient solution was renewed every 12 hours. During this time the concentration of oxygen in the root medium usually decreased from eight to five milligrammes per litre, due to the fact that the solutions were not aerated. The plants were continuously irradiated with 100 W fluorescent tubes, daylight type, about seventyfive centimetres above the plants. The temperature was $23 \pm 0.5^\circ\text{C}$.

The nutrient solution had the following composition: $\text{Ca}(\text{NO}_3)_2$ 10^{-3} M, KNO_3 10^{-3} M, MgSO_4 $5 \cdot 10^{-4}$ M, Fe^{3+} -citrate 10^{-5} M and KH_2PO_4 10^{-3} M. The pH of this solution was 5.

Experimental treatment of wheat plants. When the plants were three days old, the nutrient solution was exchanged for one which, besides the basic components mentioned above, also contained *p*-chlorophenoxyisobutyric acid (PCIB) as the potassium salt. This treatment lasted for 12 hours. In the subsequent preparations the procedure was as follows.

Extraction. The plants were removed from the nutrient solutions and the roots rapidly cut off, half a centimetre below the seeds. In some cases the roots were then immediately put into ether for extraction. In others the roots were first held in boiling distilled water for ten seconds and after that put into ether. The ether used was freed from peroxides by shaking with FeSO_4 and distillation. The extraction, which was performed at 20°C and in darkness, was made four times for half an hour each time. Two hundred millilitres of ether were used each time for approximately twenty grammes fresh weight of root material. The aliquots of ether were then added together. The ether was evaporated off from this extract at about 35°C . The residue was extracted with wet ether three times for half an hour each time. The fractions were added together and the ether evaporated off. From the residue so

obtained IAA was then extracted in a refrigerator for 12 hours by means of two applications of 4 ml. of carbon tetrachloride. The use of carbon tetrachloride turned out to be suitable for obtaining the extracted IAA in a sufficiently pure state for the following manipulation.

Chromatography. The carbon tetrachloride was evaporated off from the IAA solution at low pressure. The residue was then quantitatively transferred, by means of ether, to a strip of chromatogram paper (Munktell No 00), 2.0 cm. wide. The application was made as a spot with 2 μ l.-micropipettes. The extract applied to each strip thus originated from one treatment with approximately twenty grammes fresh weight of wheat roots. The chromatograms were run in darkness with ascending partition solvent. The solvent was composed of isopropanol, ammonia and water (10 : 1 : 1). When the solvent front had ascended about eighteen centimetres above the application spot, the strips were taken out of the solvent. The front was always allowed to move to the same height on all strips included in parallel experiments. The strips were then left to dry in darkness at room temperature.

A check strip, applied with pure IAA only, 2 μ l. of a one per cent solution, was always run parallel to the extract-strips. Another strip, with no substances applied, was run as a blank. After running, the check strip was sprayed with a solution of *p*-dimethylaminobenzaldehyde (1 g. per 100 ml. *N* HCl). This substance gives with IAA a pink-red coloured spot on the strip.

From each extract-strip a section was cut out, corresponding to the coloured spot section on the IAA-strip. A corresponding section was also isolated from the strip run as a blank.

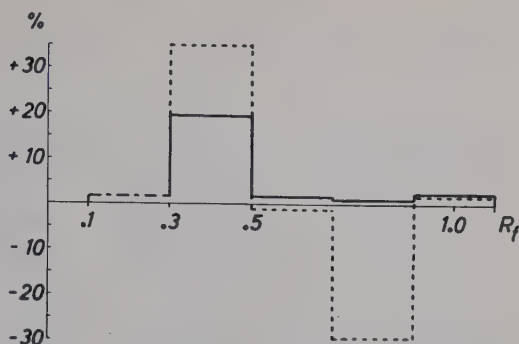
Each isolated paper section was placed along the inside wall of a 5 ml. beaker (14 mm. in diameter). The beakers were then kept for five minutes in vacuum (aqueous water pressure) at 20°C to remove residues of the partition solvent. Thereafter the amount of IAA of the extract-paper sections was measured with the *Avena* section test.

A purification of IAA and its separation from PCIB by chromatographic technique is realizable because of the rather different R_f -values of the two substances when a mixture of isopropanol, ammonia, water (10 : 1 : 1) is used as partition solvent. The R_f -values were determined by running chromatograms with the synthetic forms of the compounds. Two μ l. of 1 per cent solutions were applied to the chromatogram strips. The R_f -value of each substance was found to be constant no matter whether the two compounds were run alone or mixed. The position of IAA was detected with the reagent just mentioned. The PCIB was detected by spraying with a slightly alkaline solution of bromthymolblue, giving a distinct, yellow-coloured spot. The R_f of pure IAA usually varied little from the value 0.40 (total spot 0.29—0.51) and that of PCIB was 0.75 (0.70—0.80).

A good separation between IAA and PCIB is also obtained by chromatographic procedure when extracts from the wheat roots are used (Figure 1).

Cultivation of *Avena coleoptiles*. Seeds of Svalöv Seger oat were soaked in distilled water at room temperature for 24 hours. The seeds were then grown, with the glumes removed, in Vermiculite for three days under constant conditions, at 25°C and 80 per cent humidity. From the moment of their emergence above the Vermiculite surface the coleoptiles were irradiated for 12 hours with red light of low intensity. Thereafter the coleoptiles were kept in darkness until they were used for *Avena* section test.

Figure 1. *The effect on coleoptile section elongation of extracts from differently treated wheat roots.* — Untreated, ---- PCIB-treated roots. Extracts chromatographed and each strip cut in five parts. Each part tested in *Avena* section test. Elongation is expressed as increase or decrease in respect to control elongation.



Avena coleoptile section test. Four ml. of a basic test solution (see below) were added to each beaker containing the paper section isolated from strips earlier chromatographed. The paper was completely immersed in the solution. The composition of the basic solution was the following: potassium dihydrogen citrate 10^{-3} M (pH 4.5) and 1.6 per cent dextrose. The beakers containing paper sections and solution were left to stand for one hour and then coleoptile sections were added to the solutions.

In order to obtain good results in the test, coleoptiles were selected with lengths within rather narrow limits, usually 3.3—3.5 cm. Only one section, 5.0 mm. in length, was cut from each coleoptile. The apical 3 mm. of the coleoptiles were discarded. The primary leaf was not removed from the sections. The cutting was made by means of a double-bladed guillotine, and was carried out in red light.

Immediately after cutting, all *Avena* sections to be used in an experiment were placed together for one hour in a portion of basic solution. This was done in order to wash out the endogenous IAA from the sections and thus obtain low growth of the control. The sections were then put into the beakers containing the paper chromatogram sections to be tested. The oat sections in the beaker containing the paper section cut from the blank functioned as a control of the *Avena* test. Ten oat sections were put into each beaker. They floated on the solutions, breaking the surface. They were left in darkness at 25°C and 80 per cent humidity. After 20 hours the lengths of the sections were measured by means of a dissecting microscope with an accuracy of 0.1 mm. The average of the ten individual readings in the control was deducted from the other mean values. These differences (in hundredths mm.), divided by the dry weights of the corresponding wheat root samples, are taken as an expression of the yield of IAA obtained by the extraction.

Results

In preliminary extraction experiments the yield of IAA obtained per hour was determined. The yields varied as is reported in Figure 2. It is seen that a separate and distinct fraction of IAA is obtained during the first one and one-half to two hours. Low auxin yields are then found in the next four hours. After this time, however, the plant material begins gradually to give off

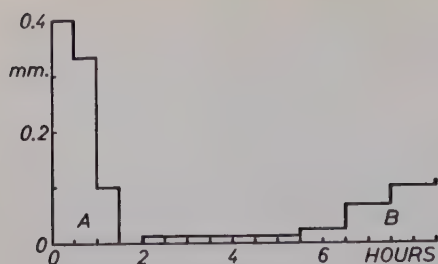


Figure 2. Rate of IAA yield from wheat roots during extraction with ether. Abscissa: the yields of IAA are presented as elongation of *Avena* sections minus the elongation of control sections. Ordinate: duration of extraction. A=IAA-fraction quickly released from both boiled and non-boiled roots. B=IAA-fraction obtained from non-boiled roots only. The fresh weight of root material extracted is 20 grammes.

IAA again. The IAA quickly released during the first hours suggests that this fraction represents what is usually called the free IAA. The IAA obtained later would then be the fraction that is released from bound IAA-complexes. Such a release, for instance by enzymatic conversion, can indubitably take place in fresh material when non-polar solvents are used (cf. 14, 22, 23).

About two hours after the start of the extraction there is evidently a period when little or no auxin is released. It seems to the present author that this period is long enough to exclude that during an extraction of short duration any important amount of IAA can originate from the bound complexes.

If the roots were kept in boiling water for ten seconds immediately before extraction with ether, no auxin was obtained after the second hour upon prolonged extraction. This would indicate that in wheat roots the conversion of IAA from bound forms is enzymatically conditioned. No destruction of the free IAA form by the heating could be observed. Gustafson (12), extracting tomato-seedlings with ether, also failed to find any destruction of auxin when the material was boiled for one minute.

The yields of short-time extractable IAA from wheat roots not treated and from those treated with 10^{-5} M PCIB are presented in Table 1. The latter root material was treated for 12 hours just before the roots were separated from the plants for extraction. It is seen from the table that the presence of PCIB causes a higher yield of IAA.

Even if there seems to be little doubt that the auxin substance isolated from the chromatograms really is IAA, the identity of the substance is proved in the following experiment. Extracts from roots not treated and from those treated with PCIB were chromatographed on separate strips as described earlier in this paper, with use of the solvent isopropanol—ammonia—water (10 : 1 : 1). From each strip that section was cut out that corresponded to the coloured spot on a sprayed check IAA-strip. The paper sections were thoroughly eluted with ether and the extracts applied to new strips. These were run with 70 per cent ethanol as the solvent. The position of the IAA spot on the check strip now was between the R_f values 0.80–0.90. The corresponding sections on the two extract-strips were then measured in the

Table 1. *The content of free, short-time extractable IAA in ether extracts obtained from wheat roots untreated and those treated with 10^{-5} M PCIB. The content is expressed as elongation in mm. of Avena sections, divided with the dry weight in grammes of extracted root material. Initial section length 5.0 mm.*

Test control, mm.	Added extract from roots not treated, mm./g. d. w.	Added extract from roots treated with 10^{-5} M PCIB, mm./g. d. w.	Roots not boiled or boiled for 10 seconds before extraction
0.45	0.59	0.75	Not boiled
0.50	0.70	0.80	"
0.39	0.49	0.63	"
0.61	0.71	0.83	"
0.87	0.97	1.06	"
0.66	0.79	0.89	"
0.52	0.67	0.75	Boiled
0.52	0.71	0.75	"
0.75	0.92	0.99	"
0.75	0.89	0.99	"
0.74	0.87	1.01	"
Average 0.61	0.76	0.86	
Difference	0.10 \pm 0.01		

Avena test for their content of IAA. The values obtained are the same as those recorded last in Table 1, for non-treated roots 0.87 and for treated ones 1.01 (mm. elongation/g.d.w.). From this experiment it is therefore concluded that the substance studied is identical with IAA.

The lengths of epidermal cells in roots treated with PCIB were found to be longer than those in untreated ones (Table 2). The lengths were estimated on cells which had elongated during the treatment for 12 hours. According to Burström (4) normal wheat root epidermal cells have an elongation-time of between fifteen and twenty hours. In both non-treated and PCIB-treated roots the highest rate of cell-stretching occurs after eight to ten hours (6).

It may be concluded that the effect of PCIB on the IAA content consists of either a competition with IAA, yielding liberation of IAA from a complex

Table 2. *The effect of PCIB on the root epidermis cell length. Length measured 12 hours after start of treatment. Temperature 23°C.*

Treatment	Cell length, in μ	Number of cells measured
Control I ...	229 \pm 5	240
PCIB 10^{-5} M I ...	319 \pm 8	240
Control II ...	218 \pm 7	240
PCIB 10^{-5} M II ...	287 \pm 7	240

of some sort, or an enhancement of the synthesis of IAA or a lowering in the rate of destruction of this substance. In favour of the first alternative is the fact that PCIB has been shown to interact competitively with IAA. Against the second alternative is the increase in cell elongation, which does not indicate that the increased IAA content is a result of reactions through those path-ways mentioned.

Discussion

The auxin level after varying treatment has been studied by some other authors. Weintraub (27) found that treatment of bean buds with 2,4-dichlorophenoxyacetic acid (2,4-D) decreased the auxin content. Henderson and Deese (15) obtained a higher IAA-content in *Avena* coleoptiles treated with 2,4-D. Kulescha (17) using Crown Gall tissue from *Scorzonera*, and Pilet (24), working with lentil roots, found no change in the IAA concentration after maleic hydrazide treatment. Audus and Tresh (3), in a careful study using chromatographic technique, observed no change of the IAA level upon treatment of leaves and roots of sunflower, bean and cabbage with 2,4-D. On 2,3,5-tri-iodobenzoic acid treatment the IAA content was strongly lowered in pea roots. However it seems to the present author that these reviewed results are rather difficult to interpret, since the different substances used in the treatments obviously have effects other than those on growth, e.g., lowering or stimulating effect on the IAA oxidase activity (10, 11, 15, 16).

The present results show that the wheat roots contain a fraction of free IAA, extractable in a rather short time, which can easily be kept apart from another fraction that is obviously enzymatically released from bound IAA forms. The concentration of the free IAA in normal roots is increased if they are treated with the antiauxin PCIB. It is thus confirmed that PCIB competes with IAA. As a result of the addition of the PCIB the root cell elongation is stimulated, a fact that was known earlier (6). It is therefore further concluded that the competition between the antiauxin and the native IAA takes place at a site where IAA exerts its elongation activity.

It is moreover apparent that some portion of IAA is already coupled to the active sites when the PCIB competition process starts. This means that the *free, non-coupled IAA is not directly active in the elongation*. Free IAA is, in fact, the form of IAA that is obtained in fraction A (Figure 2) and it is just this form that is studied in this investigation.

In wheat roots IAA obviously also exists in a bound form, a complex from which it can be released by enzyme activity; on prolonged extraction the yield of IAA is enhanced, but this does not occur by inactivating autolytic

enzymes with a brief pre-boiling of the tissue. It has been shown (Table 1) that treatment with PCIB gives a higher level of free, short-time extractable IAA whether or not the plant material is boiled before the extraction. This result does not therefore exclude the possibility that PCIB competitively releases IAA from its bound protein forms whereby IAA becomes available to promote growth. Such a hypothetical explanation was given by Skoog (25) on the action of 2,4-D. The present results, however, cannot be easily explained by means of such a hypothesis because it is apparent that IAA is released from its bound forms by the action of enzymes. It is further evident, as is discussed above, that PCIB releases IAA from a site where IAA is active in growth. *The bound forms therefore cannot reasonably be considered to represent a state in which IAA is active.* For these reasons it seems obvious that the growth-active state of IAA is something other than the IAA forms studied by the methods employed.

There are, however, a few reports that give strong evidence for the existence of a IAA form which undoubtedly represents an active state of IAA. It was presumed by Audus (1) and Audus and Shipton (2) that there exists an endogenous inhibitor in roots, which is the immediate regulator of growth. Street (26) referred the retardation of growth in subcultured tomato roots to some natural hormone which during growth accumulates to a critical inhibiting concentration. Burström (7) found that an unknown inhibiting factor limits the second phase of elongation in roots. Libbert (18, 19) reported that he had isolated from pea roots an endogenous elongation inhibitor. This is formed by a coupling between free IAA and a native inhibitor-precursor. In a recent study (20) on wheat roots Libbert concludes that the inhibitor acts at a growth centre (Libbert's second centre) which might be thought to control Burström's (4) second elongation phase. Libbert further finds that PCIB competes with IAA and impedes free IAA from coupling with the inhibitor-precursor, thereby resulting in a stimulation of the elongation. The results of Libbert and the present ones are in good accordance with each other; it is obvious from Tables 1 and 2 in the present study that the increased cell elongation obtained is mainly an effect from PCIB competing with IAA at a site where IAA acts by inhibition. It should be emphasized that only an over-all inhibiting action of IAA on the cell elongation (5, 20) has been studied in this investigation.

The question arises why the active IAA form and the inhibitor-precursor are not obtained in the preparations in the present study. Libbert (18) removed the precursor, which is easily water-soluble, by washing the roots in water. The inhibitor was then obtained by extraction of the wet roots with ether. After the addition of water to the ether-phase and a following evaporation of the ether, the inhibitor was transferred to the water-phase.

Concerning the present work a somewhat different method is used. Judging from Libbert the inhibitor can be present in the crude ether extract. In the following all of the ether is evaporated off, whereupon the dry residue is extracted with carbon tetrachloride. It is at this step unknown whether the inhibitor, in its dry form, really dissolves in the carbon tetrachloride; in any case it is observed that there is a dry portion left that does not dissolve in the solvent. It is also unknown whether the dry substance dissolves in ether during the preparation-step when the native IAA is transferred and applied to the chromatogram-strip. It is further uncertain how the inhibitor behaves during the running of the strip. Judging from the results obtained (Figure 1) the inhibitor, if present, may not dissolve into the partition solvent used. It seems therefore possible that there are at least a few reasons why the inhibitor is not obtained or cannot be demonstrated in the final preparation in this investigation.

Finally it will be emphasized that the concentration of the free IAA is not an expression of the magnitude of the growth activity of the tissue, at least not as long as a true antiauxin is present. This is also demonstrated in the following table where the present results are compared with earlier findings (9).

In the presence of a true antiauxin it is observed that:	Coleoptile length decreases	Free IAA level increases
	Root cell elongation increases	Free IAA level increases

Summary

The amount of the free, short-time extractable IAA in wheat roots was measured after treatment with the antiauxin *p*-chlorophenoxyisobutyric acid (PCIB) in a concentration of 10^{-5} M. It is found that:

1. Treatment with PCIB increases cell elongation in the roots.
2. Treatment with PCIB increases the amount of free, short-time extractable IAA.

The results are discussed and it is concluded that PCIB and IAA compete at the point of IAA action, and that PCIB acts like a true antiauxin.

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Studies in Physiological Analysis of Yield III.

The Rate of Grain Development in Wheat in Relation to Photosynthetic Surface and Soil Moisture

By

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In the earlier two papers of this series experimental evidence was presented for the existence of varietal differences in the contributions made by leaves, stem and ear to grain yield (Asana and Mani, 1950 and 1955). These varietal differences could not be accounted for completely by variation in the rate of yellowing of these organs and it was suggested that refinement in the technique of estimating the rate of yellowing could perhaps improve the correlation (Asana and Mani, 1955). Asana and Mani estimated the photosynthetic contributions from these organs to grain yield by an indirect method which involved defoliation and ear-shading and pointed out its limitations. The writers undertook, therefore, to study simultaneously the rate of increase in grain weight and the rate of yellowing of the three organs in a number of varieties of wheat, in pot as well as field culture and under adequate and deficient soil moisture. The experimental technique for such a study would obviously be simple and free from objections inherent in a technique involving defoliation and prolonged shading of ear. The object of including soil moisture as another variable was to see whether the rate of yellowing differed with variety under deficient soil moisture. Incidentally this information might be expected to throw some light on the problem of drought resistance. Observations made during two seasons are discussed in this paper.

Materials and Methods

The experiments were carried out during 1954—55 and 1955—56.

In the 1954—55 season varieties N.P. 798 and N.P. 710 were raised in glazed pots. Each pot had a capacity to hold about 35 kg. of air-dry soil, the height and diameter being 45 cm. and 22 cm. respectively. Seeds were sown on the 14th November 1954 and six uniform seedlings were ultimately retained per pot. Soil from a field under fallow in the preceding season was sieved and mixed well before filling the pots. About half a kg. of well-rotted farm-yard manure was added to every pot. Two gm. each of ammonium sulphate, superphosphate and potassium sulphate were applied to every pot about 20 days after sowing. Another 2 gm. of ammonium sulphate were applied per pot at the boot-leaf stage.

The pots were watered regularly until the 4th February 1955, but subsequently the pots under "drought" were watered only after permanent wilting occurred.

There were 35 pots under each treatment combination, 10 for final yield and the remaining 25 for sampling. The pots were kept in the open throughout, exposed to natural variation in day length and temperature.

Green leaves on one mother shoot in every pot (yield pots) were traced on paper, soon after dehiscence, (without detaching) and portions that yellowed subsequently were marked on their respective tracings at intervals of 3 to 4 days. The lengths of the stems of the same mother shoots were also measured after dehiscence and the lengths of yellow portions were noted periodically. Six mother shoots (from sample pots) were removed periodically after dehiscence and dry weights of leaves, stems, ears and grains were determined after drying at 100°C.

In 1955—56 pot and field experiments were carried out.

Varieties N.P. 718 and N.P. 710 were grown under pot-culture and the same technique as used in 1954—55 was adopted. Only 4 plants were retained per pot and the initial dose of ammonium sulphate was raised to 4 g. per pot.

The first sample was collected a week after dehiscence, which occurred in N.P. 718 on the 12th February 1956 and in N.P. 710 on the 16th February 1956, and the subsequent ones at weekly intervals. The "drought" pots were watered regularly until dehiscence and were watered subsequently only when permanent wilting occurred. 4 pots per each treatment combination were sampled on each occasion. All green leaves (excluding leaves with more than half their length yellow) were weighed quickly after detaching and a small sample of leaves was weighed separately again; outlines of the latter were traced on paper and total leaf area was calculated from the product of total fresh weight and ratio of area to fresh weight of the sample. Watering regime was so adjusted that leaves under the "drought" treatment were fully unfolded at the time of sampling. The length of the green portion of every stem was also measured. The green surface of the ear was measured by eye by adopting 4 categories of yellowness: (i) less than half yellow, (ii) half yellow, (iii) more than half yellow and (iv) fully yellow. (i) was taken as 100 % green, (ii) as 50 % green and (iii) and (iv) were taken as 100 % yellow. The percentage of green ears thus calculated in a sample was taken as an estimate of the green surface of the ear. Observations on grain number per ear and on 1000-grain weight were taken on one clump from every yield pot and on all clumps from sample pots.

Field Experiment

Varieties, N.P. 710, N.P. 718, Pb C 228 and Ridley were grown with and without irrigation in two plots separated by a strip 3 meters wide. The plot under irrigation received 2 irrigations, one on the 3rd Jan. '56 and the other on the 10th March '56. The second irrigation should have been given earlier but was delayed due to unavailability of water. A simple randomised layout with 4 replications was adopted. There were 6 rows per replicate, each 63 feet long. Sowing was done on the 4th Nov. '55 and harvesting by the middle of April '56. The first sample (12 shoots per replicate) was taken a week after dehiscence and the following ones were taken roughly at weekly intervals. The subsequent procedure was the same as already indicated for the samples from pot-culture.

A 3-foot long strip was marked out in one of the central rows in each replicate. Observations on germination, tiller number and on ear characters were taken in these strips.

Soil moisture was periodically determined up to a depth of 3 feet.

Experimental Results

Pot-Culture Experiment of 1954—55

In Figure 1 are presented the changes in time in green leaf area per mother-shoot, in green stem length, in stem weight (including leaf sheaths), and in grain weight per ear for the varieties N.P.s 798 and 710, under normal water supply (W) and under intermittent drought (D). The following points may be noted.

The green leaf area was consistently lower under D, in both varieties, thereby indicating that leaves yellowed faster under high soil-moisture tension. As the stem was still elongating when drought commenced (on 4/2/55), its growth was inhibited. This inhibiting effect was stronger on variety N.P. 710 as it eared slightly later than N.P. 798 (dehiscence in N.P. 710 on 16/2/55 and in N.P. 798 on 12/2/55). The leaves yellowed more quickly than the stem. Under W the dry weight of the stem increased and/or remained constant for about 3 weeks after dehiscence and then it decreased gradually, whereas under D the decrease in stem weight commenced earlier. The rate of decrease in stem weight was more or less parallel under W and D after 3 weeks from dehiscence.

It is remarkable that for the first 4 weeks or so after dehiscence the grain weight increased at a similar rate under W as well as D, although the leaf area was consistently lower under the latter. The grain weight under W exceeded that under D only during the last week or week and a half, when little of green leaf or stem surface was left.

Under W the leaf and stem surfaces of N.P. 798 were slightly but consistently higher than those of N.P. 710, but the grain weight increased at

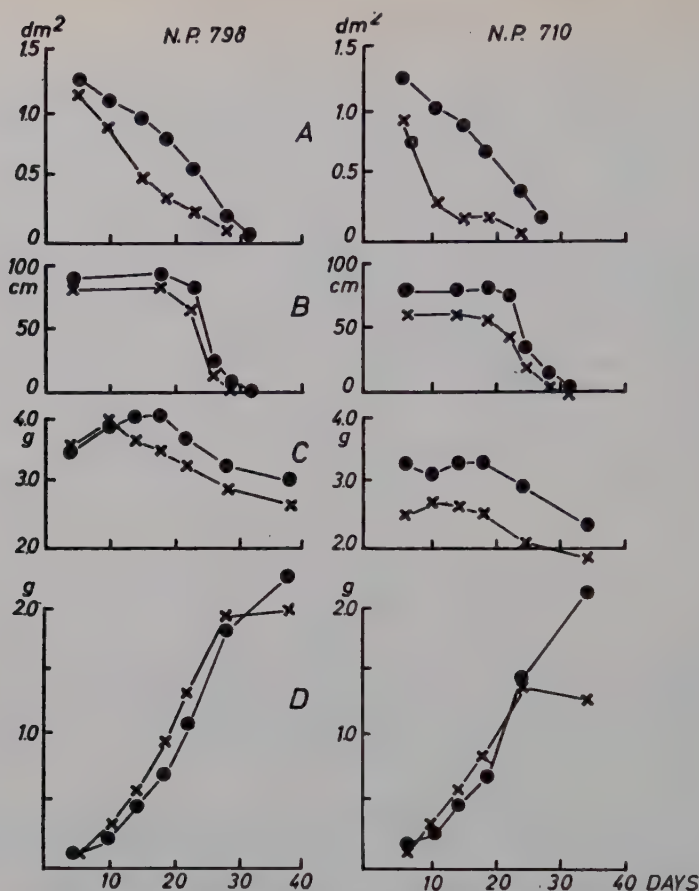


Figure 1. Abscissa: Days after dehiscence. ●—● Normal water supply. ×—× intermittent drought. A, green leaf area per shoot. B, green stem height. C, dry weight of stem. D, grain weight per ear.

a more or less similar rate in both. The difference between the two varieties in respect of leaf and stem surfaces was larger under D, but the grain weight increased at a similar rate during the first 24 days. Subsequently there was little increase in grain weight in N.P. 710, while it was appreciable in N.P. 798. After 24 days from dehiscence the leaf and stem surfaces of N.P. 798 were slightly larger than those of N.P. 710, but it appears doubtful, in view of their magnitude, whether these contributed materially to grain development in the former. Perhaps the ear might have remained green longer in N.P. 798.

The mean grain weight per ear from the yield pots is compared with the final mean grain weight per ear (mother shoot) from the sample pots, in

Table 1. *Mean grain yield per ear in g.*

Material	N. P. 798		N. P. 710	
	W	D	W	D
Yield pots	1.70	1.47	1.51	1.10
Sample pots (mother shoots)	2.28	2.00	2.10	1.31

Table 1. The grain weight from the yield pots was lower because ears from mother shoots and tillers were included. The differences due to variety and treatment are consistent in both cases, thus indicating that the difference between the grain weights at the last sampling date, due to differential water supply and variety, was real.

The grain yield of N.P. 798 was much higher than that of N.P. 710 under D than under W. As the former dehiscence earlier data on temperature and evaporation during the post-dehiscence period were examined but no appreciable differences were found.

An estimate of effective leaf and stem surface, during the post-dehiscence period, was obtained by determining the area enclosed by the leaf surface and stem height curves (leaf and stem indices). These data are included in Table 2, from which it appears that differences in grain yield, due to variety or water level, were poorly associated with difference in leaf or stem index.

Pot-Culture Experiment of 1955—56

In the preceding season observations were made on mother shoots only. During 1955—56 observations were made on whole clumps and four pots under each treatment combination were sampled on each occasion. In 1954—55 the drought treatment was started on 4/2/55, whereas in 1955—56 it was started after dehiscence. The rate of yellowing of ear was also estimated.

Table 2. *Comparison of grain yield with leaf and stem indices.*

Variety	Grain yield in g. per pot			Leaf Index sq. dm. days			Stem Index cm. days		
	W	D	% Df. ¹	W	D	% Df.	W	D	% Df.
N. P. 798	99.2	74.2	26	19.7	11.5	42	1940	1620	17
N. P. 710	94.2	62.8	34	15.5	4.5	71	1490	1060	29
% Df.	5	16	—	22	35	—	23	35	—

¹ Percentage Difference.

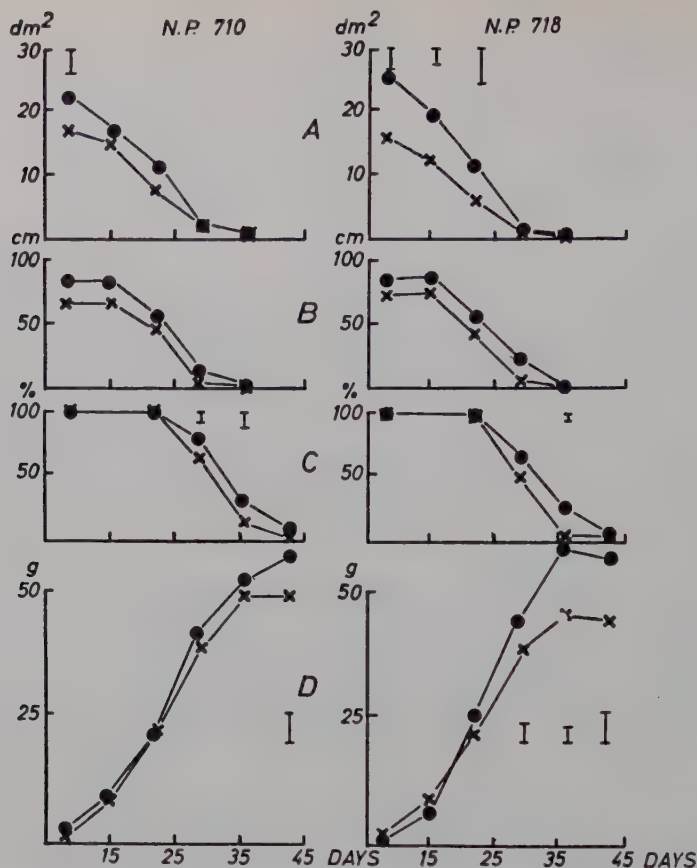


Figure 2. Abscissa: Days after dehiscence. ●-● Normal water supply. ×-× intermittent drought-t. A, green leaf area per pot. B, green stem length. C, green surface of ear. D, grain weight per pot. I, significant difference at 5 % P.

In Figure 2 are presented the changes in time in green leaf area per pot, in green stem length, in green surface of ear and in grain weight per pot. The data were statistically analysed (analysis of variance) and the critical differences at 5 per cent probability are indicated wherever necessary.

During the first 21 days, the grain weight in the two varieties increased at a similar rate under W and D, the green surface of leaves and stems was consistently lower under D, while the ear remained completely green under W and D. There was a substantial increase in grain weight in the two varieties under W and D after 29 days, by which time the leaves and stem had yellowed almost completely and this increase appears to be associated with the (partially) green surface of the ear. The larger increase in grain

Table 3. *Data on grain yield, ear characters and leaf and stem indices.*

Variety	Grain yield per pot in g.			Grains per ear		1000-grain weight in g. Days after dehiscence						Leaf Index Sq. dm. days		Stem Index cm. days	
	W	D	% 1 Df.	W	D	21		29		Final		W	D	W	D
N. P. 710	57.1	48.8	15	44.0	37.1	17.4	18.4	27.7	30.8	35.7	36.5	279	225	1420	1070
C. D. at 5 % P (W & D)	4.0			3.2		0.96		N. S. ²		N. S.		—	—	—	—
N. P. 718	57.8	45.3	22	45.8	35.7	17.1	19.5	27.4	32.0	34.6	36.2	348	186	1640	1240
C. D. (W & D)	1.97			2.8		1.2		0.1		0.9		—	—	—	—
C. D. (Varieties)	N. S.	0.9	—	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.	—	—	—	—
% Df.	—	8	—	—	—	—	—	—	—	—	—	21	18	15	16

¹ Percentage Difference.² Not significant.

C.D.=Critical difference.

Table 4. *Soil moisture as percentage of oven-dry soil.*

Date	Unirrigated plot (I ₀)			Irrigated plot (I)		
	Depth 0-1'	1-2'		0-1'	1-2'	
		2-3'			2-3'	
22/11/55	11.6	14.1	15.4	12.6	14.8	15.3
5/12/55	8.1	13.2	14.6	9.6	13.7	14.8
20/12/55	7.6	11.7	13.6	7.8	10.8	13.2
13/1/56	8.1	10.3	11.3	17.6	17.2	15.8
8/2/56	5.3	8.4	10.4	8.0	11.8	12.4
3/3/56	4.9	7.6	8.8	5.8	8.6	9.4
1/4/56	4.4	7.9	8.9	7.6	10.4	8.9
Moisture equivalent	14.9	17.5	19.3	1st irrigation: 3/1/56		
Wilting coefficient	4.7	7.2	7.3	2nd " 10/3/56		

weight under W in the two varieties, after 29 days, is also apparently associated with the relatively larger green surface of the ear.

Data in Table 3 indicate that grain number was reduced under D, whereas the final 1000-grain weight was not appreciably affected by D. It is interesting to note that the 1000-grain weight was slightly but consistently larger under D up to 29 days after dehiscence; apparently with the commencement of drought grain setting was adversely affected, but as the ear remained equally green (as under W) the grain size was increased more than under W. Later when the ear yellowed faster under D, the increase in grain size slowed down and thus the ultimate 1000-grain weight was more or less equalised.

Under D N.P. 710 outyielded N.P. 718 by a small margin of 6 per cent. The larger increase in grain weight in N.P. 710 between 29 and 36 days was apparently associated with the relatively larger green surface of the ear. The differences between the varieties in respect of leaf and stem surface were not consistent. There was lack of consistent correlation between leaf and stem indices and grain yield.

Field Experiment of 1955—56

Moisture stress commenced in the first 2 feet of the unirrigated plot (I_0) from early February and in the third foot from early March (Table 4). The stress increased further towards the end of March as indicated by partially folded leaves. In the irrigated plot (I) the crop was subjected to some stress from the third week of February to the first week of March on account of delay in the second irrigation. The data on variety N.P. 710 alone are presented in Figure 3; those on the other varieties are not considered because of an unconscious bias in the samples from the I_0 plot. Up to the first 30 days the grain weights were similar in I_0 and I, whereas the green leaf and stem surfaces were consistently larger in the latter. There was little increase in grain weight in I_0 subsequently. On the 30th day, of the original leaf, stem and ear surface, there remained 28, 54, and 96 % respectively in I and 0.27 and 50 % in I_0 . The longer persistence of functional ear

Table 5. *Mean 1000-grain weight in g.*

Irrigation	Days after dehiscence				
	21	30	34	38	44
(I)	13.4	29.6	32.6	37.4	38.5
(I_0)	15.4	29.5	33.6	35.2	35.2
C. D. (5 % P)	N. S.	N. S.	N. S.	1.15	1.15

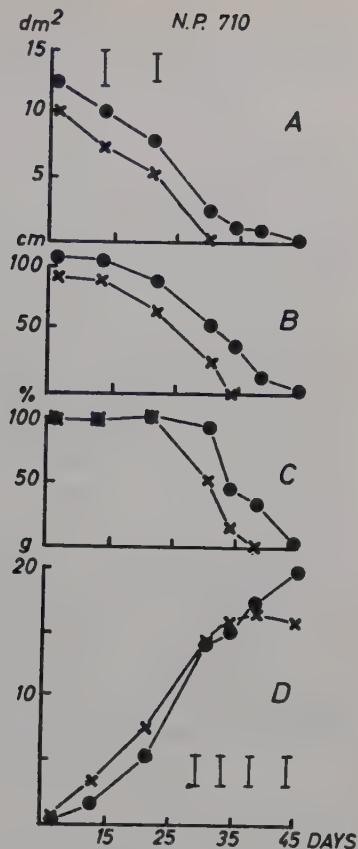


Figure 3. *Abscissa: days after dehiscence.* ●-● irrigated. ×-× unirrigated. A, leaf area per 12 shoots. B, green stem length. C, green surface of ear. D, grain weight per 12 shoots. I, significant difference at 5 % P.

surface very probably accounted for the ultimate increase in grain weight in I. The grain number per ear in I_0 and I did not differ significantly. The 1000-grain weight increased at a more or less constant rate in I_0 and I up to 34 days, but subsequently it was higher in I (Table 5). The ear number per plant was not higher in I probably because of absence of timely irrigation during the critical period and thus its higher yield per unit area was due to higher yield per ear.

Discussion

It was seen that under drought, during the first four weeks or so of the post-dehiscence period, yellowing of leaves and stems was hastened, the rate of increased in grain weight was not affected, the grain number was

reduced and the 1000-grain weight was increased. After leaves and stem yellowed, the rate of increase in grain weight was larger under normal water supply due to greater increase in 1000-grain weight, this increase being associated with slower yellowing of ear. It would seem that photosynthesis in the ear played an important role in grain development. The supply of material from the stem was perhaps not so important in view of the fact that grains under normal water supply alone increased in weight during the last week although the loss in stem weight was more or less equal under the two water treatments. The importance of the ear in grain development has been demonstrated by Porter *et al.* (1950) and denied by Gabrielsen (1942). The former recorded assimilation by day and respiration by night, of the ear of barley, under normal outdoor conditions, whereas the latter made these observations, on the basis of fresh weight, on detached ears of wheat under artificial light. Thorne and Watson (1955) and Watson (1956) concluded, however, from their experiments on time of application of nitrogen, that the tail of the leaf area growth curve was directly concerned in grain production in wheat. Those workers appreciated, however, the importance of the ear, although they did not estimate its functional surface.

Since the varietal difference in yield was found to be associated with the rate of yellowing of ear, it would seem worthwhile to explore the extent of variation in this character, with a view ultimately to aid breeding of varieties suitable for unirrigated conditions.

Summary

The rate of increase in grain weight was studied in three varieties of wheat in relation to photosynthetic surface and soil moisture. The plants under pot culture were subjected to intermittent drought (watering after permanent wilting), a little before or after dehiscence, as soil moisture stress generally becomes acute, under unirrigated cultivation in this country, during this stage of development.

Under drought during the first four weeks or so of the postdehiscence period, yellowing of leaves and stems was hastened, the rate of increase in grain weight was not affected, the grain number was reduced and the 1000-grain weight was increased. After leaves and stems yellowed, the rate of increase in grain weight was larger under normal water supply due to greater increase in 1000-grain weight, this increase being associated with slower yellowing of ear. As photosynthesis in the ear apparently played an important role in grain development, it is suggested that the extent of variability in the rate of yellowing of ear should be further examined with a view ultimately to aid breeding of varieties suitable for unirrigated conditions.

The authors record their thanks to Dr. B. P. Pal, Director, and Dr. S. M. Sikka, Head of the Botany Division, for their encouragement in the prosecution of these studies.

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**Studies in Physiological Analysis of Yield IV.
The Influence of Soil Drought on Grain Development,
Photosynthetic Surface and Water Content of Wheat**

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It was reported in paper III of this series (Asana *et al.* 1958) that variation in green leaf area and stem surface, during the post-dehiscence period, either due to drought or variety, had little influence on the rate of increase in grain weight so long as the ear remained green. Grain weight increased even after the leaves and stems yellowed and this increase in grain weight was dependent upon the rate of yellowing of the ear. The bearing of this observation on breeding for drought resistance in wheat was indicated and the desirability of exploring the range of variability in the rate of yellowing of ear was pointed out. During 1956—57 the experiment was repeated with two other varieties of wheat and the water content of the different organs was also determined. The main findings, reported earlier, were confirmed and some additional information was also obtained. These observations together with a fuller discussion on their bearing upon the problem of drought resistance are presented in this paper.

Materials and Methods

Two varieties of wheat, N.P. 720 and Pb C 281, were raised in glazed pots according to the technique described earlier (Asana *et al.* 1958). The pots were watered regularly until the 4th February 1957 and subsequently the pots under

'Drought' were watered only when permanent wilting occurred. There were 15 pots (each with 5 plants) under each treatment combination for final yield and 90 for sampling. The date of dehiscence in every mother shoot in each pot was carefully recorded and only mother shoots were sampled for study in the interest of precision of data. In general Pb C 281 dehisced earlier than N.P. 720 by 4 days (N.P. 720 by the 18th February 1957 and Pb C 281 by the 14th Feb.). Sixty mother shoots were cut off at ground level, on each sampling occasion, from 15 pots under each treatment combination and were divided into 4 lots (replicates) of 15 shoots each. There was provision for six samples to be collected at approximately weekly intervals, but due to early maturity only five samples could be collected. The fifth sample could not, however, be collected in its entirety because of damage by a severe hailstorm on the 20th March 1957. The watering regime was so adjusted that on each sampling occasion the plants under 'drought' were permanently wilted, these plants being watered after sampling was over.

As it was intended to determine the water content of green leaf tissue, laminae yellowed beyond half their length and dry and yellow portions of the other leaves were discarded. After taking fresh weights, the different parts of the shoot were dried in an oven at 100°C and weighed. The rate of yellowing of ear was estimated in the manner described earlier (Asana *et al.* 1958). The data were analysed statistically (analysis of variance) and the significant differences are indicated by symbol S wherever necessary in the figures.

Experimental Results

The changes in time in grain weight per shoot, in stem weight, in ear surface and in leaf number of variety N.P. 720, under normal water supply (W) and drought (D), are presented in Figure 1. For sample V, only 30 shoots, each, were available from sample pots of W and D. These were divided at random into two lots of 15 each. Under W the mean grain weight per shoot from the two lots was 2.29 and 2.44 g and under D 2.12 and 2.14 g. Intact mother shoots from the 15 yield pots were also harvested from each pot. The mean grain weight per shoot under W and D was 2.34 and 2.06 g. respectively (C. D. at 5 per cent P; 0.17 for 28 degrees of freedom). The agreement between the values from sample and yield pots is good and the latter values were inserted for sample V in Figure 1.

The difference between grain weights under W and D was significant only at samples 3 and 5. We may conclude that the grain weight increased at a more or less similar rate under W and D upto 28 days after dehiscence, in spite of appreciable difference in green leaf surface (leaf number). The leaves yellowed completely by 28 days and subsequently grain weight increased significantly only under W. After 21 days the ear began to yellow under both W and D but at a significantly faster rate under D, with the result, that a week later nearly half its surface was yellow as against only

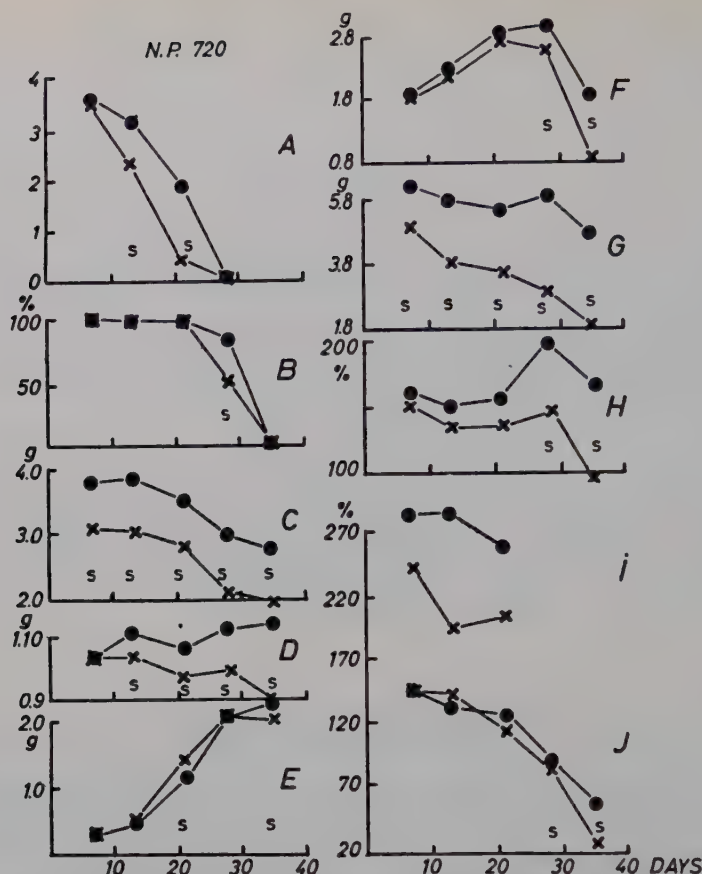


Figure 1. Abscissa: days after dehiscence. ●-● normal water supply. ×-× intermittent drought. A, leaf number per shoot. B, green ear surface. C, dry weight of stem. D, dry weight of chaff. E, grain weight per ear. F and G, water content of ear and stem respectively, H, I and J, water content of stem leaf, and ear, respectively, as percentage of dry weight. S, significant difference at 5 % P.

11 per cent under W. The stem weighed significantly less under D throughout. The stem weight remained constant for the first 13 days and then declined gradually.

Analysis of variance of the dry weight of awns, rachis, glumes etc. (chaff) of the first four samples indicated that the interaction of water treatment and time was significant at 5 % P. The dry weights of samples II, III and IV of W were significantly higher than those of D. The dry weights of samples II, IV and V (2 replicates only) were significantly higher than that of sample I under W, thus the chaff weight under W increased between 7 and 13 days after dehiscence and then remained constant. As the dry weight of samples I, II, III and IV under D did not differ significantly among themselves and were higher than those of sample V,

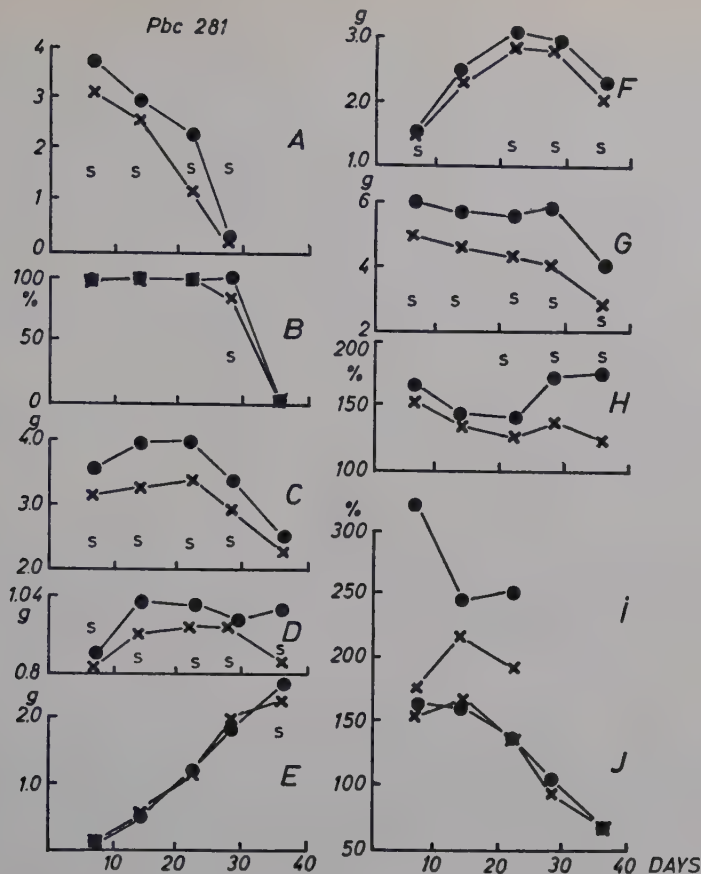


Figure 2. Same legends as for Figure 1.

it follows that the dry matter of chaff under D remained more or less constant for the first 28 days after dehiscence and then decreased.

In Pb C 281, the same trends in the changes in time of grain weight, leaf area, stem weight and of ear surface can be noticed as in N.P. 720 (Figure 3). Only 15 intact shoots, each, were available from sample pots under W and D and the mean grain weight per shoot varied as 2.50 and 2.40 g. The mean grain weight per intact shoot from yield pots was 2.62 g. for W and 2.28 for D (C.D., at 5 per cent P, 0.14 for 28 degrees of freedom). The latter values for sample V are inserted in Figure 3.

The effect of water treatment and time were significant on the dry matter of chaff, thus indicating that it was consistently lower under D, that it decreased between 7 and 14 days under W and then remained constant up to 28 days after dehiscence in both W and D. After 28 days it probably remained constant under W and decreased under D.

Water content.

The changes in the water content of the various organs of N.P. 720 are shown in Figure 2. The water content of the ear, either on percentage or absolute basis, was not affected by drought during the first 21 days. In Pb C 281 (Figure 4), the absolute water content of the ear was slightly lower under D throughout, whereas the percentage water content was not affected. The absolute water content of the ear increased steadily until the first 3 weeks, was then maintained at more or less the same level for another week and then declined. Between 21 and 28 days the ear begins to turn yellow but it is difficult to trace any causal connexion between water content and yellowing.

The absolute water content of the stem was larger under W primarily because of the greater length; the percentage water content was, however, lower under D only during the later stages.

The water content of the leaves of both varieties was reduced considerably under D, the leaves having permanently wilted at the time of sampling. Among the three organs, the water content of the leaves was the most severely affected by drought, while that of the ear was the least affected.

Comparison of the two varieties

The grain weight of the two varieties increased under W and D at a more or less similar rate until 4 weeks after dehiscence. Subsequently the rate of increase was distinctly higher in Pb C 281. The leaf area, as judged by number, was occasionally larger in Pb C 281, but the grain weights were more or less similar until the time the leaves yellowed completely. Again the difference in ear surface appeared to be associated with the ultimate difference between the grain weights of the two varieties.

The percentage water content of the ear of Pb C 281 was consistently higher than that of N.P. 720 under W and D; yellowing of the ear commenced at about the same time in both and there does not appear to be any causal connexion between water content and yellowing.

Grain number per ear and 1000-grain weight.

The data on grain number per ear in the various samples are given in Table 1. In both varieties the effect of water alone was significant, thus indicating that grain setting was adversely affected by drought. The significant effects of time and of interaction of time and water treatment in N.P. 720 indicated that grain number was lower in the intermediate samples.

Table 1. *Mean grain number per ear.*

Variety	Treatment	Days after dehiscence				
		7	13	21	28	Mean
N.P. 720	W	63.0	61.5	60.2	62.0	61.7
	D	59.7	56.2	58.7	58.5	58.3
	Mean	61.4	58.9	59.5	60.2	
		C.D. (5 % P): water treatment: 0.94 » : time : 1.3 » : interaction : 1.6				
Pb C 281	W	7	14	22	28	Mean
	W	57.7	61.5	61.5	59.2	60.0
	D	57.5	56.5	58.3	58.7	57.7
	Mean	57.6	59.0	59.9	59.0	
		C.D. (5 % P): water treatment: 1.6				

It is not possible to account for the interaction effect but the main conclusion about the adverse effect of drought on grain number is not affected thereby.

The data on 1000-grain weight are given in Table 2. The 1000-grain weight was generally higher under D for the first 28 days; only subsequently did it increase much more under W than under D. Pb C 281 led over N.P. 720 only during the last week; it may be recalled that the grain weight of N.P. C 281 exceeded that of N.P. 720, under W as well as D, only between 4 and 5 weeks after dehiscence.

Due to damage caused by the hail-storm the data on grain yield per pot were not reliable.

Table 2. *Mean 1000-grain weight in g.*

Variety	Treatment	Days after dehiscence				
		7	13	21	28	Harvest
N.P. 720	W	4.2	7.4	20.0	33.4	42.6
	D	4.0	8.9	23.7	36.8	40.6
	C.D. (5 % P)	—	1.1	2.0	1.0	1.4
		7	14	22	28	Harvest
Pb C 281	W	2.8	7.8	20.2	31.9	47.2
	D	3.1	9.2	20.3	34.8	45.4
	C.D. (5 % P)	—	0.9	—	1.7	N.S.

Discussion

The results reported earlier (Asana *et al.* 1958) and those reported in this paper have consistently shown that intermittent drought, during the post-ear emergence period, inhibited stem growth, hastened yellowing of leaves and stems and reduced grain number. In spite of these adverse effects, the grain size (1000-grain weight) was increased and thus the same rate of increase in grain weight was maintained under D (as in W) during the first 3 to 4 weeks. Subsequently the ear began to turn yellow, and at a faster rate under D, the leaves and stems yellowed completely under both water treatments, and the rate of increase in grain size and the final grain yield were depressed under D. It was suggested that photosynthesis in the ear played a major role in grain development, because the ear alone remained completely green during the first 3 to 4 weeks when grain development was not affected by D, whereas it was depressed subsequently when the ear yellowed faster. Although Porter *et al.* (1950) have experimentally shown the importance of photosynthesis in the ear in grain development in barley, this interpretation must be considered in relation to current ideas on 'hardening' and the effect of soil moisture on photosynthesis and catabolic processes in leaves.

Induction of 'hardening' by drought has been shown to enhance capacity for photosynthesis in leaves (Tumanov, 1927 — quoted by Maximov, 1938). This effect was, however, seen in new 'xeromorphic' leaves; in our experiments new leaves did not develop after drought was started and therefore 'hardening' cannot account for the same rate of grain development under drought during the first four weeks.

Opinion on the influence of soil moisture stress on photosynthesis in leaves is divided. According to Allmendinger *et al.* (1943), Loustalot (1945), and Upchurch *et al.* (1955), the rate of photosynthesis is little affected until the permanent wilting percentage is closely approached, while according to Schneider and Childers (1941), Bourdeau (1954) and Ashton (1956), it decreases even before wilting is evident. Even if we accept the first view, total photosynthesis must decrease due to reduction in leaf area under drought and thus affect grain-filling.

According to Maximov (1938), transport of catabolic products out of leaves increases under drought; this increase might compensate for decrease in photosynthetic supply from leaves. Losses in stem weight under W and D were, however, found to be more or less similar about a fortnight after dehiscence. In the 1954—55 experiment (Asana *et al.* 1958) the losses in leaf weight were 0.10 and 0.13 g. under W and D, respectively, in N.P. 798 and 0.21 and 0.13 g. under W and D, respectively, in N.P. 710. Thus there was

little consistent evidence to show that catabolic products from leaves and stem sufficiently compensated for decreased photosynthetic supply under D.

The senior author (Asana, 1957) has discussed elsewhere the problem of soil drought as it affects wheat, under Indian conditions. With the data obtained since then the perspective has become more clear and the rest of the discussion will be devoted to this matter.

It is notheworthy that the ear had the same water content under the two water treatments. The reduction in grain number is, therefore, puzzling. It is conceivable that the deficit, in water content of the flowers was not detected when the overall water content of the ear was determined. Grain setting might have been adversely affected due to interference with fertilization processes due to moisture deficit in flowers or due to reduction in supply of nutritive substances, probably nitrogenous in nature, from rapidly senescing leaves.

Drought after ear emergence affects yield by reducing the number and size of grains. The size is apparently controlled by the rate of yellowing of ear but the grain number may be determined by a number of factors. In our case, grain setting was reduced by drought, the number of flowers being already fixed. It would be useful to establish whether varieties are differentially influenced by drought in regard to grain setting. It is conceivable that varieties A and B may suffer the same percentage reduction in grain number under drought and yet a larger grain number may set in A by virtue of its larger flower number. The absolute grain number would thus appear to be a crucial factor in the determination of yield.

Asana *et al.* (1955) reported that ear number had the most potent effect on grain yield under normal water supply, while grain number had the same effect as ear number, and 1000-grain weight a comparatively smaller effect on yield under drought; the 1000-grain weight was, however, found to be relatively the most stable character. They recommended that grain number per ear and 1000-grain weight should be given due weightage while selecting varieties for unirrigated conditions. We can now add that high grain number and an ear with a longer functional period would be desirable attributes for unirrigated conditions. Incidentally spraying ears with substances that delay their yellowing might be suggested as a possible means of increasing yield under such conditions.

Summary

Experiments with two other varieties of wheat confirmed the earlier observation (Asana *et al.* 1958) that reduction in leaf and stem surface, due to drought after ear emergence, did not affect grain development as long

as the ear remained green. Drought reduced the grain number of the two varieties to the same extent. The 1000-grain weight of Pb C 281, and therefore the yield, exceeded that of the N.P. 720, under normal water supply and drought, only during the last week of the maturation period when the ear of the former was relatively greener and the leaves and stems of both were yellow. It is pointed out that the little effect of drought on grain development during the early stages cannot be accounted for by increase photosynthetic activity due to 'hardening' or by increase in supply of catabolic products from leaves.

Under drought the water content of the ear was little affected but the grain number was reduced; this anomaly may perhaps be due to failure to detect minute changes in the water content of flowers. The bearing of these observations on breeding varieties for unirrigated conditions is discussed.

The authors wish to record their thanks to Dr. B. P. Pal, Director of the Institute, and Dr. S. M. Sikka, Head of the Botany Division, for their continued inspiration and interest in the progress of these investigations.

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The Effect of 2,4-Dinitrophenol on Respiration, Oxydative Assimilation, and Photosynthesis in *Chlorella*

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The effect of 2,4-dinitrophenol (DNP) on various metabolic properties of *Chlorella* has been studied already by a number of authors (Gaffron 1942, Holzer 1951, 1954, Kessler 1955, Kandler 1955, Kandler und Frank 1956). Probably the most exciting findings are those by Gaffron (1942) and Holzer (1951, 1954) which serve as an evidence for the participation of energy-rich phosphates in photosynthesis. It was the purpose of this work to find out to what extent photosynthesis is a DNP-sensitive process.

Material and Methods

The same strain of *Chlorella pyrenoidosa* was used as in earlier works (Holzer 1951, Kandler 1955). The algae were cultivated in 1 l. gas-wash-bottels with an illumination of about 5,000 Lux on the glass surface. The mineral solution was the same as in another paper (Kandler 1951). The algae grew at a day length of 16 hrs. Three hrs. after the light was switched on in the morning, the suspensions were harvested by centrifugation and used for the experiments about one h. after they were removed from the light.

All manometrical measurements were done in the usual way. Glucose was determined according to Folin and Wu (see Kandler 1954); inorganic phosphate according to Martland and Robison (see Kandler 1951).

To determine the radioactivity 50 mm.³ of the suspension were placed on an aluminium plate of 4 cm.² and counted in a methan flow counter.

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Experiments and Results

a) Respiration and oxydative assimilation of glucose

2 ml. of *Chlorella* suspended in a 1/30 *M* phosphate buffer of pH 5.6 were pipetted into a manometer flask, and 0.5 ml. DNP and glucose-solution and water, respectively, were added to give a final concentration of glucose of 0.5 % or of DNP corresponding to the concentrations in Table 1. After measuring the O_2 -uptake for a period of 3 hrs. an aliquot of the samples containing glucose was taken and the residual glucose determined. The data of a typical experiment are shown in Table 1. In contrast to other objects, like yeast (Lynen and Koenigsberger 1951), eggs of sea urchins (Clowes and Crahl 1936), carrot tissue (Kandler 1950) etc., where respiration is increased by a factor of 2 to 10, our *Chlorella* strain shows only an increase in the endogenous respiration by about 50 %. Concentrations higher than $0.5 \cdot 10^{-4}$ *M* inhibit the respiration. The RQ is not changed considerably as shown recently (Kandler 1958).

In the presence of glucose, DNP does not lead to a significant increase in respiration. Concentrations increasing the endogenous respiration maximally, do not affect glucose respiration, but as soon as the optimal concentration is surpassed, a strong inhibition takes place. The oxydative assimilation of glucose is already inhibited by low concentrations and the ratio O_2 -uptake: assimilated glucose decreases continuously with increasing DNP concentration. Again in contrast to other tissues, there is no complete oxydation of glucose. The inhibition of respiration is too strong and leads to the death of the algae, before the point of complete uncoupling is reached.

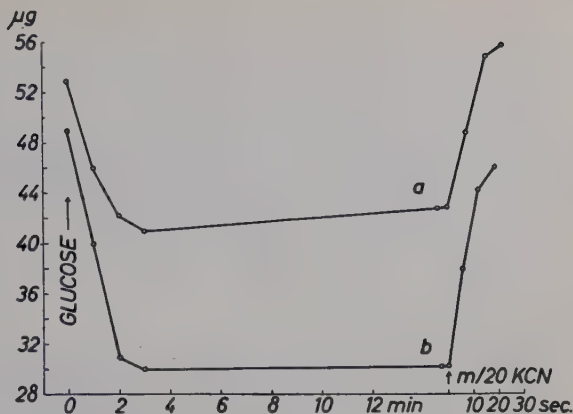
b) Rate of phosphorylation

As far as we know now, the increase in respiration by DNP is caused by the uncoupling of oxydative phosphorylation, thus preventing the control

Table 1. Oxygen and glucose uptake of 2 ml. *Chlorella* suspension under the influence of different DNP-concentrations. pH=5.6. 1/30 *M* phosphate buffer. Gas phase: air; $t=27^\circ C$.

Concentration DNP molar	Without substrate O_2 mm ³ /h.	+ Glucose O_2 mm ³ /h.	Δ Glucose mg./h.	O_2 mm ³ /mg. glucose
0	23	86	0.98	88
10^{-4}	22	25	0.066	380
$0.5 \cdot 10^{-4}$	35	82	0.4	205
$0.25 \cdot 10^{-4}$	36	90	0.76	118
$0.125 \cdot 10^{-4}$	31	86	0.9	95.5
$0.062 \cdot 10^{-4}$	28	87	0.93	94

Figure 1. Influence of DNP on the changes of the level of inorganic phosphate in *Chlorella* cells after adding glucose and KCN, respectively. On the ordinate PO_4^{3-} $\mu\text{g/ml}$. a: with DNP b: control.



of the respiration rate by the amount of phosphate acceptors or inorganic phosphate, respectively. To get an idea of the degree of inhibition of phosphorylation processes, we can use the decrease in glucose assimilation, which is inhibited by about 25–30 % at the maximal stimulation of endogenous respiration. A much more direct method is the one Lynen and Koenigsberger (1951) used in their experiments with yeast. The experiments presented in Figure 1 are based on their ideas.

A suspension of *Chlorella* cells in distilled water was divided into two parts. After adding DNP in a concentration, which causes maximal respiration (tested manometrically) to one flask, both samples were shaken at 20°C for 1 h. in order to give a constant action of DNP. Then a 2 ml. sample was put in a manometer flask, with the sidearm containing 0.5 ml of a 5 % glucose solution, and the O_2 -uptake was measured. From the large sample in the Erlenmeyer flask, 3 samples, 5 ml each, were withdrawn and given immediately into 6 % TCA (end concentration). Glucose was then added to a final concentration of 1 % and further samples were withdrawn in the following minutes. After 15 min. KCN was added to a final concentration of 1/20 M and samples were withdrawn as quickly as possible. The same was done with both samples. After 5' min. extraction of the algae in TCA, they were centrifuged, washed twice with 6 % TCA and the inorganic phosphate in the collected extract was determined. At the same moment as the glucose was added, also in the manometer flasks, glucose from the sidearm was added to the main vessel and readings were made every 5 min.

In agreement with Lynen's und Koenigsberger's experiments with yeast, DNP leads to an increase in inorganic phosphate in the steady state of endogenous respiration. When glucose is added to the sample with DNP the decrease in the inorganic phosphate is slower and the new level is not as low as in the control sample. If KCN is added, the increase in inorganic phosphate with DNP is not as steep as in the control. Following the assumption of Lynen and Koenigsberger, the rate of phosphate liberation after KCN

poisoning is the same as the phosphorylation rate was before the addition of KCN. Thus the rate of phosphorylation can be computed from such an experiment. Together with the O_2 -uptake, measured manometrically, we are able to compute P/O ratios. For the control we obtained a value of 1.78, but for the DNP sample we find only 1.14. Thus DNP inhibits phosphorylation by about 30 %. The same figure was obtained by basing our computation on the inhibition of the glucose uptake.

c) *Photosynthesis*

When measuring the effect of DNP on the photosynthetic CO_2 -uptake, it is very difficult to keep the pH constant. While CO_2 is taken up, the pH is shifted more and more to the alkaline side. On the other hand, the action of DNP is extremely sensitive to the pH and decreases strongly with increasing pH. In order to obtain an exact coordination of the effect of DNP on respiration and oxydative assimilation on one side and photosynthesis on the other, the following experiments were carried out.

2 ml. of a *Chlorella* suspension in 1/30 M phosphate/HCl buffer, pH = 2.7, were put into manometer flasks and 0.5 ml. DNP and glucose solution added. The side-arm contained 0.5 ml. of 1/10 M $KHC^{14}O_3$ (about $2\mu C$). The vessels were placed in the Warburg apparatus, equipped with a very strong high pressure Xenon lamp for high, or with fluorescence tubes for low light intensities. Some of the vessels were surrounded by a metal case with a red glass bottom, only transmitting light of a wave-length longer than 560 m μ . The arrangement of the lamps has been described more fully by Kandler und Schötz (1956). To arrive an idea of, in what part of the light saturation curve we are working, the same algal material was suspended in a bicarbonate/carbonate buffer (95/5) and given into vessels of the same shape as used in the DNP experiment, but shielded to different degrees by screens. Then the samples were put in the Warburg apparatus and the O_2 -production was measured for 1 h. The weak light from the fluorescent lamps gave about 50 % saturation, the red light 100 % and the full intensity of the white light from the Xenon lamp led to a 50 % inhibition. Thus we obtained 3 very different parts of the saturation curve.

After placing the vessels with DNP in the thermostate, the manometers were closed and the radioactive bicarbonate was added. After mixing, the solution had a pH of about 5.6, but after photosynthesis for 3 h. the pH increased to about 6.2. After 3 h. the suspension was taken out and the residual glucose was determined. While low light intensities favour the glucose uptake of starved cells (Kandler 1954, Simonis 1955), it is inhibited strongly by high light intensities (Sironval and Kandler 1958). As shown in Table 2 a, DNP leads to an additional inhibition by about 50 % at a concentration of 10^{-4} M. But at the same time photosynthesis is only very slightly blocked, as indicated by the radioactivity of the algae; at lower

Table 2a. $C^{14}O_2$ Fixation and glucose uptake of 2 ml. *Chlorella* suspension under the influence of different DNP-concentrations. 1/30 M phosphate buffer pH 5.5 to 6.2. Gas phase: air + $C^{14}O_2$; $t=27^\circ C$.

DNP molar	100,000 Lux white light				50,000 Lux red light				8,000 Lux white light			
	CO ₂ Fixation		Δ Glucose/h.		CO ₂ Fixation		Δ Glucose/h.		CO ₂ Fixation		Δ Glucose/	
	CPM/ml.	%	mg.	%	CPM/ml.	%	mg.	%	CPM/ml.	%	mg.	%
0	1,500	100	0.5	100	2,700	100	0.9	100	1,450	100	1.2	100
10^{-4}	1,250	83	0.15	30	2,550	94	0.4	44	1,300	90	0.55	45
$5 \cdot 10^{-4}$	1,450	96.5	0.25	50	2,710	100.5	0.6	67	1,430	98.5	0.85	70

concentrations still causing 30 % inhibition of the glucose uptake, there is no inhibition of the photosynthesis at all.

In another experiment only one light-intensity of about 8000 Lux, was applied but 3 different DNP concentrations, each in 3 parallel samples. As shown in Table 2 b, an inhibition of glucose uptake up to 50 % does not influence the photosynthesis, but a further increase of the DNP-concentration leads to an extreme inhibition of the photosynthesis as well.

To make sure, that this striking stability of the photosynthesis against DNP-poisoning is really true, another type of experiments was carried out.

A dense *Chlorella* suspension (1/100 M phosphate buffer, pH 5.6) was divided in several portions and DNP at different concentrations was added. 2 ml. of each sample was put into manometer flasks and one out of two parallel samples was supplied with glucose to a final concentration of 0.5 %. For 3 hrs., endogenous and glucose respiration were measured. At the end, the glucose uptake was determined in the usual way. The rest of the original samples was shaken on a shaker, until it was used for the photosynthesis experiments. They were done in the following way: 3 ml. of the suspension were put in a lollipop (Calvin and Massini 1952)) with a water jacket to maintain the suspension at $15^\circ C$. The light sources consisted of two 500 Watt lamps, the infrared was filtered off by water. After illumination for 5 min. in a mixture of air and 1 % CO_2 , 0.2 ml. of 1/100 M $KHC^{14}O_3$ (30 μ C) was injected by a syringe. After 15, 30, or 60 sec. respectively, the suspension was dropped into boiling alcohol. After measuring the volume of the suspension plus alcohol, 3 aliquots were plated and counted.

Table 2b. Conditions as in Table 2a but a different batch of *Chlorella*. Average of 3 parallel samples.

DNP	CO ₂ Fixation		Δ Glucose/h.	
	CPM/ml.	%	mg.	%
0	1,700	100	0.78	100
$2 \cdot 10^{-4}$	450	26.5	0.17	22
10^{-4}	1,630	96	0.37	47.5
$0.5 \cdot 10^{-4}$	1,710	100.8	0.55	71

Table 3 a. *Oxygen and glucose uptake of 2 ml. Chlorella suspension under the influence of different DNP-concentrations. No buffer; pH about 5.8; t=27°C; CO₂-fixation was measured by short time fixation of C¹⁴O₂ in a "lollipop". Light saturation.*

Concentration DNP molar	Without substrate O ₂ mm ³ /h.	+ Glucose O ₂ mm ³ /h.	Δ Glucose mg./h.	O ₂ mm ³ /mg. glucose	CO ₂ - Fixation (C PM · 10 ³ /ml.)		
					15 sec.	30 sec.	60 sec.
0	30	90	0.92	98	405	1195	3400
10 ⁻⁴	41	79	0.4	197	403	1080	3000
0.5 · 10 ⁻⁴	43	87	0.55	158	390	1145	3240
0.25 · 10 ⁻⁴	42	86	0.61	141	395	1060	2990
0.125 · 10 ⁻⁴	37	91	0.78	116	400	1105	3250

Table 3 a presents the results of such an experiment. In agreement with the experiment described above, there is no considerable inhibition of photosynthesis, even if the glucose uptake is inhibited by 50 %.

A second experiment, using a higher concentration, is illustrated in Table 3 b. It indicates again, that only very high concentrations of DNP affect photosynthesis as well.

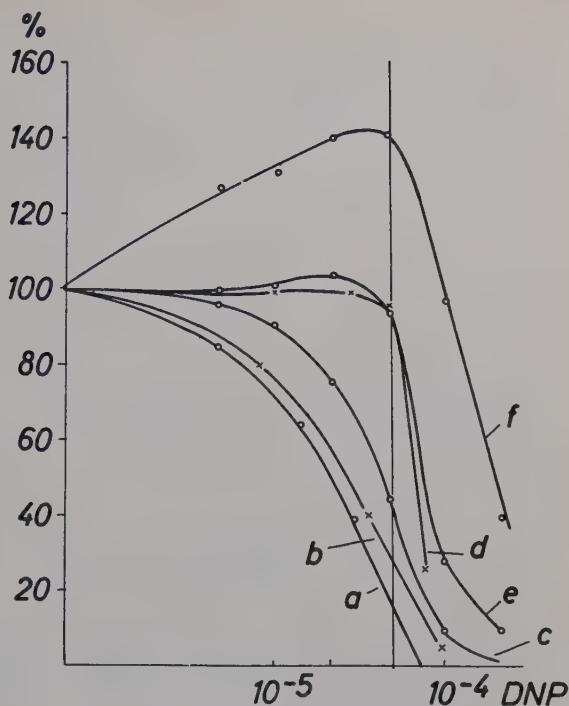
Discussion

As all the described experiments are done with the same material, one can compare exactly the influence of DNP on respiration and assimilation processes. In addition to the data mentioned in the tables above. Figure 2 contains also data from an earlier paper (Kandler and Frank 1956) on the effect of DNP on ammonia assimilation of the same *Chlorella* strain and on nitrite reduction by *Ankistrodesmus* (Kessler 1955). We can distinguish two types of curves in Figure 2. The first type, to which the curves for glucose and ammonia assimilation and nitrite reduction belong, shows a continuous drop with increasing DNP concentration. The second type,

Table 3 b. *Same as 3 a but a different batch of Chlorella was used.*

DNP molar	Without substrate O ₂ mm ³ /h.	+ Glucose O ₂ mm ³ /h.	Δ Glucose mg./h.	O ₂ mm ³ /mg. glucose	CO ₂ - Fixation CPM · 10 ³ /ml.		
					15 sec.	30 sec.	60 sec.
0	45	143	1.65	87	1120	3200	9900
2.5 · 10 ⁻⁴	28	59	0.25	235	360	720	1450
1.25 · 10 ⁻⁴	55	132	0.80	165	1110	2900	9400
0.6 · 10 ⁻⁴	65	150	1.6	94	—	—	—

Figure 2. Effect of different DNP-concentrations on the various metabolic processes of *Chlorella*, expressed in % of the control. a: ammonia assimilation; b: nitrite reduction; c: glucose assimilation; d: photosynthesis; e: glucose respiration; f: endogenous respiration. (Curve for nitrite reduction according to Kessler 1955).



including respiration and photosynthesis, shows a sudden change at a concentration of $0.5 \cdot 10^{-4} M$, where both processes are very strongly inhibited, while lower concentrations are not effective, nor even stimulating.

The effect of the low concentration up to $0.5 \cdot 10^{-4} M$ is easily explained on the basis of the mechanisms discussed by Lynen and Koenigsberger. The uncoupling of the oxydative phosphorylation to a certain degree (in the case of *Chlorella* by about 30 %) leads to an increase in respiration up to a level, where processes, other than phosphorylation, are limiting. For *Chlorella* this level is obviously about 150 % of the endogenous respiration. In the presence of glucose the phosphate turnover is already increased by the glucose assimilation (see Kandler 1958) and the respiration is limited by other factors. Thus, DNP has no influence. The uncoupling of oxydative phosphorylation leads to an inhibition of the ATP-requiring processes *e.g.*, ammonium and glucose assimilation, or nitrite reduction, the first being the most sensitive one.

The complete insensitivity of CO_2 fixation with in this low range is surprising. According to Calvin's hypothesis (Calvin and Bassham 1946), 3 ATP are needed for the reduction of 1 CO_2 and the ATP is produced by recomb-

nation via the cytochrome-system. Thus, a very high sensitivity against DNP should be expected.

If the DNP concentration is increased further, endogenous respiration is inhibited returning to the level without DNP, and finally drops to zero. Simultaneously, also glucose respiration decreases and photosynthesis shows a strong inhibition. This is in agreement with Holzer's experiments. He also used high concentrations when he observed marked inhibition of the photosynthesis. Stating that photosynthesis is inhibited already at concentrations not changing the respiration, Holzer worked with concentrations already depressing the respiration to the level of the endogenous one.

Holzer's results have often been interpreted (Arnon 1956) to show the necessity of ATP for the reduction of CO_2 . According to the present data, it seems doubtful whether this holds true. We know, that the specific uncoupling effect of DNP is the activation of endogenous respiration and the inhibition of oxydative phosphorylation at low concentrations, but we do not know what mechanism is responsible for the inhibition of respiration at high concentrations. The fact, that the uncoupling of phosphorylation is by far not complete, when the inhibition of respiration starts, ($\text{O}_2/\text{mg. glucose} = 205 \text{ mm}^3$, complete oxydation should yield 750 mm^3 .) indicates the occurrence of a second, quite different effect of DNP. On the basis of the data presented here, we would like to suggest, that photosynthesis is insensitive to the typical uncoupling effect of DNP, but very sensitive to an unknown action of DNP, by which respiration is inhibited as well.

If energy-rich phosphate is necessary for photosynthesis, it has to be formed by an DNP insensitive mechanism and not by recombination of electrons via systems used by Arnon (1956) in his early experiments with chloroplasts. Indirect indications for such a DNP insensitive mechanism can be found in the data of Kandler (1955) and more direct evidence in the recent observations of Arnon (1957), who found ATP formation simultaneously with the reduction of TPN.

Summary

The effects of DNP on endogenous and glucose respiration, as well as on oxydative assimilation of glucose and ammonia, and on photosynthetic CO_2 -uptake are all measured on the same *Chlorella* suspension. It is shown, that DNP concentrations, which inhibit the oxydative phosphorylation up to 50 % and increase endogenous respiration maximally, do not inhibit

photosynthesis. Only concentrations inhibiting respiration and acting presumably by a second, still unknown type of mechanism, also inhibit photosynthesis. It is suggested that a cytochrome-like system for the generation of ATP cannot be involved in the photosynthesis. Light phosphorylation is assumed to be DNP-insensitive.

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Growth Substances in the Roots of *Pisum sativum*

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In root growth the nature of the endogenous, hormonal, growth-controlling factors is still under dispute. The role of IAA is a particular enigma, since difficulties arise in interpreting the effects of externally applied growth regulators (Audus and Shipton 1952, Audus and Das 1955, Åberg 1957). There is considerable indirect evidence for the participation of non-auxin growth inhibitors (Audus and Brownbridge, 1957).

Auxin chromatography has revealed the presence of several growth promoting and inhibiting substances in extracts of root tissues. The results of Britton, Housley and Bentley (1956) on tomato roots, and of Housley, Booth and Phillips (1956) on maize roots, have demonstrated that in these materials IAA is present in very low quantities relative to the growth promoting activity found in the water soluble fractions of their extracts; this activity cannot be due to IAA, and may indicate a non-indole auxin system.

The present work extends the observations of Audus and Thresh (1956) on the growth substances of pea (*Pisum sativum*) roots by the use of other assay techniques and by examination of other fractions of root extracts.

Material and Methods

Materials: Extracts were prepared from seedling roots of *Pisum sativum* var. Meteor, grown in sand for three days, and then transferred to water culture. The

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roots were grown in virtual darkness, while the shoots were lit by fluorescent lamps, controlled to a 14 hour daylength. The temperature was 25°C. throughout.

Extraction: — The cold alcohol method (Kefford 1955) was used. For each chromatogram, 30–60 gm. of roots were harvested, frozen at –15°C., macerated in chilled absolute ethanol, and left at –15°C. in darkness for about 18 hours. The extract was then filtered under suction, and the filtrate concentrated and purified in various ways as subsequently detailed. During all these operations, exposure to strong light was avoided.

Chromatography: In general, the methods advocated by Nitsch (1956) were followed, using 2 cm. wide strips of Whatman No. 2 paper, and iso-butanol : methanol : water: 80 : 5 : 15 as solvent. After development, the chromatograms were removed from the tanks, dried in still air, and divided into 20 transverse strips, the first including the whole of the starting spot, and the last passing slightly beyond the solvent front, which, in general, travelled about 25 cms.

The Bioassays: The small strips were each eluted into 1.0 ml. of bioassay medium (10^{-2} M potassium monohydrogen phosphate, 0.5×10^{-2} M citric acid, 2 % sucrose) in 1"×1" diameter glass tubes (Audus and Thresh 1953), by cutting them into about 10 small pieces and immersing them in the 1.0 ml. of medium some 3 hours before the coleoptile or mesocotyl segments were added. Under these conditions the paper itself had no inhibitory effect on the response of segments to IAA.

(i) *Avena* mesocotyl assay: Oats, var. Victory, grown in moist sand, were harvested after 72 hours in *complete darkness* at 25°C. Segments, 3.1 mm. in length, were excised after discarding the coleoptile and the apical 2.0 mm. of the mesocotyl. After pre-soaking for one hour on muslin stretched across the surface of glass distilled water, they were distributed at random amongst the eluates to be assayed.

(ii) *Avena* coleoptile assay: 3.1 mm. segments were excised after discarding 3.0 mm. of coleoptile tip from seedlings grown as above, except that they were given a two minute exposure to light, 56 hours or so after sowing. This had the effect of reducing mesocotyl and stimulating coleoptile growth.

(iii) *Wheat* coleoptile assay: Segments were excised as in (ii), from seedlings grown as in (i).

In all cases, the manipulations were carried out in light from a Kodak yellow-green filter.

Samples of ten sections were used in each assay, and straight growth was recorded photographically after 18 hours' gentle agitation, in darkness, at 25°C.

Control segments were grown in basal medium containing a strip of Whatman No. 2 paper; 10 such tubes, each with 10 segments, were normally included for *each* set of chromatograms in any one assay. Growth in length was calculated as a percentage of the mean of these ten controls. Fiducial limits calculated from the standard deviations of the 10 control results were taken as the criterion of significant deviations indicative of active spots on the chromatograms. On the accompanying histograms, these significant deviations are black.

Results

I. Acetonitrile fraction

Several series of extracts were treated with acetonitrile using the method employed by Nitsch (1955–1956). The filtered ethanolic extract, after

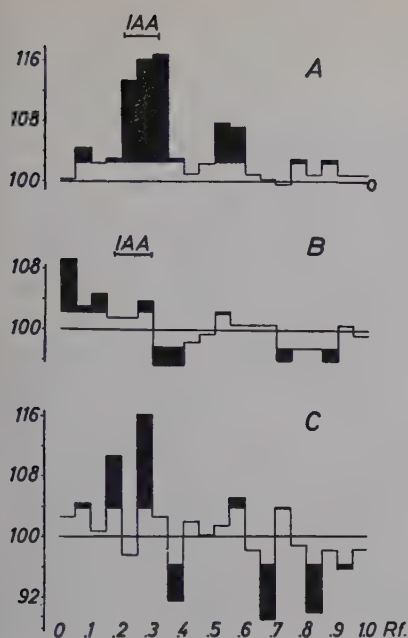


Figure 1.

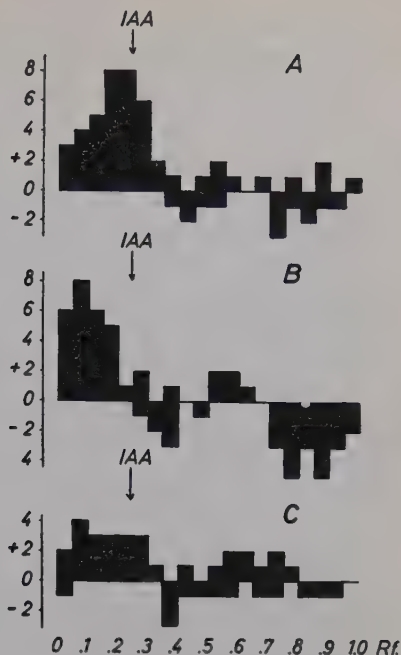


Figure 2.

Figure 1. *Chromatograms of acetonitrile-soluble fraction of ethanol extracts. (Growth responses plotted against Rf. values.)*

A. — *Avena mesocotyl test.*

B. — *Avena coleoptile test.*

C. — *Wheat coleoptile test.*

Figure 2. *Diagram showing integrated results of all analyses on acetonitrile-soluble fractions of ethanol extracts. (Number of significant responses plotted against Rf. values.)*

A. — *Avena mesocotyl tests.*

B. — *Avena coleoptile tests.*

C. — *Wheat coleoptile tests.*

+ = Spots showing significant growth promotion.

— = Spots showing significant growth inhibition.

concentration under reduced pressure to a syrup, was extracted with five successive portions of acetonitrile, and these partitioned against hexane and again concentrated under reduced pressure. The residue was then taken up in ether (Analar grade) for application to the chromatogram strip. At no time did the temperature rise above 35°C.

According to Nitsch (1956), this method is quantitative for IAA, and indole-3-acetonitrile (IAN). The present work could not substantiate these claims since known amounts of IAA added to the frozen roots immediately

before maceration could not be recovered quantitatively on the chromatograms. Nevertheless certain qualitative results of interest were obtained.

Sample histograms illustrating the kind of result obtained with the different assay methods are shown in Figure 1. These and other similar chromatograms are summarised diagrammatically in Figure 2. Here, for each assay method, on each occasion when a significant response was obtained, it has been scored in its appropriate Rf. position either above (promotion) or below (inhibition) a line representing the control level of section elongation. Each square therefore represents one significant response.

All three assay methods revealed a zone of growth promotion below the IAA position (AP(i); $R_f=0-0.15$). Here, and subsequently, "A" refers to acetonitrile purification, "P" to promotor and "I" to inhibitor). It was especially noticeable with the *Avena* coleoptile (Figure 1 B); rather less so with the wheat (Figure 1 C), and still less so with the mesocotyl tests (Figure 1 A). On the other hand, the mesocotyl sections were apparently more sensitive to activity at the IAA position, (AP(ii); $R_f=0.15-0.3$) just as they are, in fact, more sensitive to IAA itself. Activity at these two positions was also found in the acid fraction of the ether soluble material in extracts purified according to the bicarbonate-phosphoric acid procedure, as used by Audus and Thresh (1956).

A zone of inhibition occurred slightly above the IAA position (AI(i); R_f varying from 0.3 to 0.5). The greater magnitude of the effect in the wheat test (Figure 1 C) is almost certainly related to the much greater elongation of the tissue in the controls. This zone was also present in chromatograms of the acid fraction, *i. e.* it probably corresponds to the "inhibitor β " of other workers (*e.g.* Kefford 1955).

Between R_f 0.5 and 0.6, both *Avena* tests indicate the presence of a substance which is slightly promoting at the concentrations present on the chromatograms (AP(iii)). Results with the wheat assay are less conclusive. Since rather scattered inhibitions were recorded over this range in all three tests, but especially with the wheat, it seems probable that in many of the chromatograms, this promoter is partially or completely masked by growth retarding material.

Similar interference of inhibitory and promoting substances seems to occur at higher R_f values. The tests consistently indicate a broad zone of inhibition above the region of R_f 0.7 (AI(ii)), particularly with the *Avena* coleoptile. However, the test which is most sensitive to growth stimulation by IAA, and least sensitive to inhibitors *i.e.* mesocotyl section growth, reveals the presence of promoting activity throughout most of this same zone. It is notable also that in many of the coleoptile assays, the inhibition was split

into two zones, as though it had been offset by a promoter occupying the central part of its Rf. range (Figures 1 B and C). In no case, however, did the "promotion" reach significance with these tests.

II. *The Water-Soluble, Ether-Insoluble Fraction*

The use of prechromatography in water for purification (*cf.* Nitsch 1955, Housley and Bentley 1956), showed that, although water soluble material could be separated from water insoluble substances, which remained at the starting line, large quantities of impurity remained in this water soluble fraction. This impurity was inhibitory over most of the length of the water chromatograms, but when eluates were re-chromatographed in iso-butanol, methanol, water, it did not spread above Rf. 0.5. At higher Rf.-values the chromatogram was clean and was very growth active. When concentrated eluates of water chromatograms were extracted with various solvents, it was found that comparatively pure preparations could be obtained. Ethanol was the most useful, in that it removed only a small amount of impurity. Methanol dissolved much more impurity; acetonitrile and ethyl acetate both gave very clean extracts with, however, very little growth activity; n-butanol was even less effective.

The procedure finally adopted is illustrated by the following typical experiment. 600 gms. roots from 9-day old pea seedlings were harvested, extracted and the ethanol distilled off under reduced pressure, leaving 190 ml. of aqueous residue at pH 5.5. This was divided into three equal portions, each of which was shaken out five times with equal volumes of Analar ether. To complete the removal of ether solubles, the aqueous residues were acidified to pH 3 with sulphuric acid, and once again partitioned against ether.

The ether washings were combined and purified by initial chromatography in water and then chromatographed in iso-butanol, methanol, water. The activity present was of a very low order. The active zones compared closely with those in acetonitrile extracts with the exceptions of the premotor at Rf 0.5—0.65.

The acidified aqueous residues were neutralised with barium hydroxide, filtered, and concentrated under reduced pressure to give 9.74 gm. of a brown syrup, specific gravity 1.27. The syrup was re-dissolved in 4.0 ml. glass distilled water, aliquots extracted overnight at 2°C. in 2.0 ml. absolute ethanol (or, in experiments illustrated in Figures 3 A and B, ethyl acetate and acetonitrile, respectively), and these extracts concentrated and applied to chromatogram strips as starting spots. A 0.1 ml. aliquot of the concentrate was equivalent to 5.4 gm. of the original root tissue.

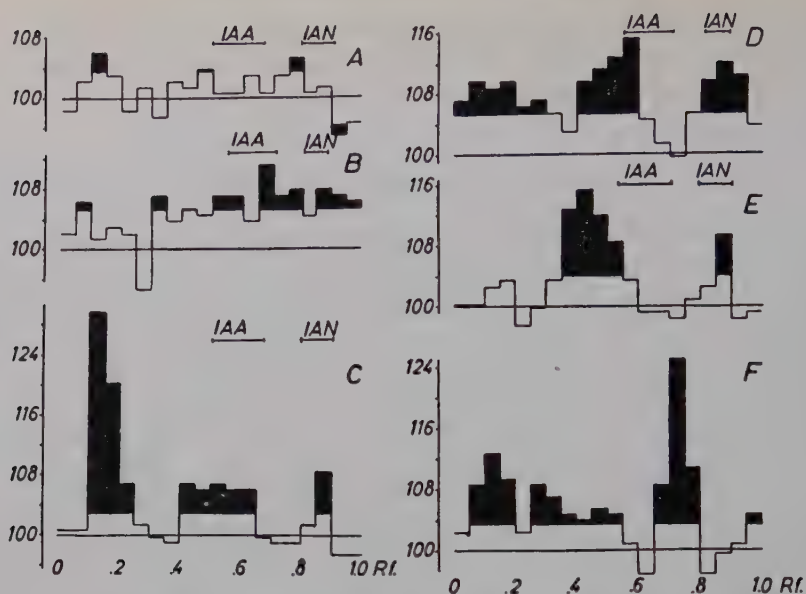


Figure 3. Chromatograms of water-soluble, ether-insoluble fractions of ethanol extracts. (Growth responses plotted against Rf. values.)

A. — Ethyl acetate-soluble.
 B. — Acetonitrile-soluble.
 C. to F. — Ethanol-soluble.

Direct assays of such chromatograms are shown in Figure 3 A—F. In general, when ethanol was used in the final purification (as in C—F), three zones of activity appear. For convenience (following the previously used system, "P" refers to growth promotion and "W" to water solubility), these will be designated as WP (i), WP (ii), and WP (iii). The series of chromatograms shown were prepared at varying time intervals after the roots were harvested, and, when this factor is taken into account, it can be seen (Table 1) that there is a fairly rapid loss of activity during storage at -15°C . in darkness.

Table 1. Loss of activity in the water soluble, ether insoluble fraction.

Expt. No.	IAA equivalent ($\mu\text{g./100 gm. tissue}$)				Time (days) from harvesting of roots to commencement of assay
	WP (i)	WP (ii)	WP (iii)	Totals	
40 (Figure 3C)	8.5	0.9	0.3	9.7	11
45 (Figure 3F)	1.1	0.9	3.3	5.8	14
42 (Figure 3D)	0.4	0.5	0.3	1.2	28
44 (Figure 3E)	—	0.3	0.04	0.34	35

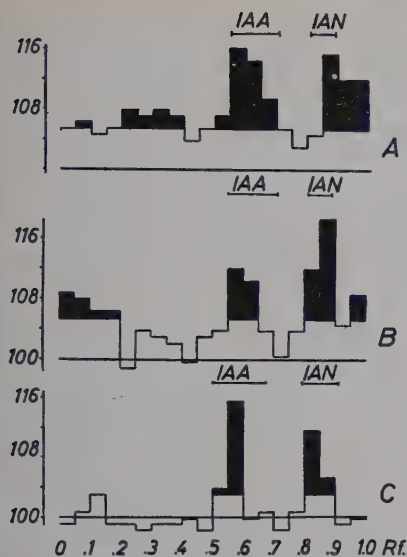


Figure 4. Chromatograms from the eluates of a primary chromatogram of the water-soluble, ether insoluble fraction of an ethanol extract. (Growth responses plotted against Rf. values.)

- A. — Eluate from WP (i) zone; Rf=0.05—0.25.
 B. — Eluate from WP (ii) zone; Rf=0.45—0.65.
 C. — Eluate from WP (iii) zone; Rf=0.75—0.95.

The similarity of the chromatograms to those of Britton, Housley and Bentley (1956), prepared from tomato-roots, prompted an investigation of the behaviour of the zones when eluted and re-chromatographed. Appropriate sections of chromatograms were selected, cut into small pieces, eluted for 3 hours at 2°C. in 2.0 ml. absolute ethanol, and the eluates concentrated and re-chromatographed in the same solvent system. Typical eluates from the three zones are shown in Figure 4. Clearly each substance breaks down and gives rise to activity at its own and the other two positions.

In general, the chromatograms of the eluates show clear separation of well defined peaks. This suggested that the interconversion does not occur during the actual chromatographic separation. Evidence that interconversion is not restricted to the process of elution was obtained from the experiment illustrated in Figure 5. In it, a water soluble concentrate, purified through ethanol, and equivalent to 12.6 gm. root tissue, was chromatographed, first along one axis of a sheet of paper (A→B), and then, after drying, along the

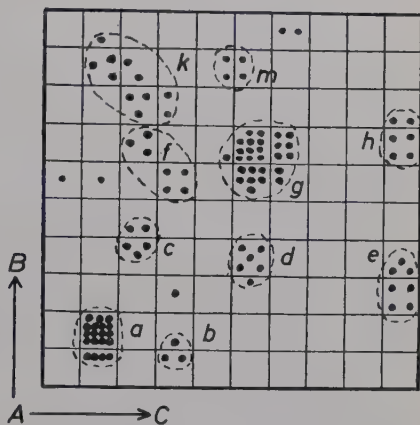


Figure 5. Two-way chromatogram of the water-soluble, ether-insoluble fraction of ethanol extract (for explanation 'see text').

second axis (A→C). A control, developed along the AB dimension only was included (Fig. 3 F). For bioassay, the two-dimensional chromatogram was cut into ten strips, and each strip into ten segments. In the figure those portions of the chromatogram causing a growth of the coleoptile segments significantly above controls are spotted, *each* spot in *each* square representing a one percent increase in growth above the fiducial limits. The interpretation of the spot groupings is somewhat subjective but after careful inspection and comparison with one-way chromatograms the probable positions of active substances have been outlined with dotted lines.

In addition to WP (i) (Rf. 0.1 to 0.2), WP (ii) (Rf. 0.5 to 0.7) and WP (iii) (Rf. 0.8 to 1.0) a fourth substance makes its appearance at Rf. 0.2 to 0.4 [Called now WP (iv)]. It would seem that at some time between the first and second running the following interconversions have taken place. WP (i) (spot a) has remained very largely unchanged except for the production of a very small quantity of WP (iv) (spot b). WP (ii) (spot g) also remained largely unchanged except for production of small quantities of WP (iii) (spot h) and WP (iv) (spot f). WP (iii) however entirely disappeared in this interval giving rise to WP (i) (spot k) and WP (ii) (spot m). WP (iv) (spot c) was also partially converted to WP (ii) (spot d) and WP (iii) (spot e). Now that the greater separation made possible by this two-way chromatography has shown up the presence of WP (iv), substance can be detected on earlier one-way chromatograms *e.g.* Figure 3 F, 4 A and 4 B, although significance levels are not always reached.

Several attempts were made to obtain colour reactions between these water soluble substances and the ferric chloride — perchloric acid reagent (prepared as in Gordon and Weber 1951, but diluted by half with ethanol). No positive results were obtained, even on inspection in ultraviolet light (Linser and Kiermayer 1956) and with very active material. This does not necessarily imply the absence of indole nuclei in the substances since a similar extract to which 25 μ g. of IAA had been added, when chromatographed and sprayed, likewise gave no positive colour reactions. On the other hand, IAN in precisely similar conditions gave a typical reaction. Obviously, substances in the extract must interfere with the colour reaction of IAA but not IAN.

Discussion

Ether soluble growth substances

The growth substances separated by purification through acetonitrile and hexane can probably be directly compared with those which have previously been found in the acid fraction of ether soluble material.

Activity at an Rf. lower than IAA occurs in extracts of numerous tissues, including pea roots (Kefford 1955, Cartwright *et al.* 1956, Audus and Thresh 1956, and Pate 1956). Early suggestions that the substance responsible was indolepyruvic acid (Stowe and Thimann 1954) have been questioned (Bentley *et al.* 1956). Instead, Housley and Bentley (1956) conclude that one such substance, the "accelerator α " of Bennet-Clark and Kefford (1953), is an artefact, produced during purification by the effects of heat and bicarbonate on a neutral, water-soluble precursor. This system cannot account for the AP (i) activity described in this paper, since the extracts were never exposed to any alkali, and were only mildly warmed. It might possibly be indole-acetylaspatic acid, which occupies a similar Rf. range (Good *et al.* 1956), is active in the *Avena* straight growth test, and can at least be formed in pea roots from added IAA (Andreae and Good 1955), but it is insoluble in ether except under very acid conditions (confirmed by Dannenberg and Liverman 1957). Britton, Housley and Bentley (1956) suspect that two of the water soluble auxins from tomato roots can, in certain conditions, be found in the ether soluble fraction. The histogram of Figure 3 B shows that some water-soluble growth activity can be extracted in acetonitrile, yet, on solubility grounds, IAA cannot be involved, although there is activity at its position. For these reasons, AP (ii) cannot be automatically assumed to be IAA on Rf values alone.

The other zones may be dealt with briefly. Promotion at the AP (iii) position, *i.e.* between IAA and IAN, has not previously been recorded for pea roots, although it has been found in several other tissues, *i.e.* wheat roots (Lexander 1953), potato peelings (Blommaert 1954), maize kernels (Stowe and Thimann 1954), green cabbage and spinach ("E" thought to be indoleglycollic acid by Fischer (1954); compare Bentley *et al.* (1956), cauliflower heads (Kefford 1955), blackcurrant ("Ribes 2") (Wright 1956) and apple ("Malus 2") (Luckwill 1957).

Audus and Thresh (1956) and Cartwright *et al.* (1956) have recorded promotion at or around the AP (iv) position. Its rather erratic appearance, as in the present work, is in line with the more extensive results of Pate (1956), on growth substances in pea root nodules, and confirms his conclusions that the variability comes from interaction of a promotor and an inhibitor.

Ether insoluble, water soluble growth substances

There are some similarities between WP (i), WP (ii), and WP (iii) and some labile substances found in cabbage extracts (Housley and Bentley 1956), *e.g.* the water soluble precursor at the Rf. of IAA which liberates IAN under the influence of alkali, and the water soluble precursor of accelerator α .

These, however, are unlikely to be the substances on the chromatograms here described, (a) because they were never treated with any alkali and (b) because breakdown can occur without any heating whatsoever, see *e.g.* Figure 5.

Resemblances to the system of water soluble auxins in tomato roots (Britton *et al.* 1956) and maize coleoptiles and roots (Housley, Booth and Phillips 1956) are very much stronger. The tomato root X and Y zones each gave rise to X, Y, and Z peaks when eluted and re-chromatographed. Z gave rise to a spot which might have been due to either X or Y or both, but its behavior was not examined except after exposure to bicarbonate. Here the fact that correspondence occurs between the Rf. values of the active zones in the eluates and in the primary chromatograms in *at least three* solvent systems is good evidence that the results do represent actual interconversions and not merely coincidences. It follows from this that although three distinct, chromatographically separable substances must exist, it is possible that only one of them, or a metabolic breakdown product thereof, is growth active, *i.e.* activity at the three zones could result from conversion of each to a common active substance either spontaneously, or by metabolic reactions in the tissue used for bio-assay. This active material could be IAA.

In several experiments, two identical chromatograms were prepared, and one of these stored while certain zones of the other were eluted and re-chromatographed; both were then assayed directly. In such cases quantitative comparisons can be made between the amount of activity in the eluates and the amount in the original zone. Because the validity of using an IAA calibration curve to obtain quantitative data on unknown substances is questionable, since the concentration-response curve may be quite different, the following conclusions can only be very tentative. Nevertheless the eluate usually contains more total activity than the original zone (see typical experiment in Table 2). It is of interest that in the paper by Britton *et al.* (1956) an exactly similar increase in activity is shown in Fig. 2 A (Zone Y eluted) and Figure 2 C (corresponding eluate) of the water soluble auxins of tomato roots. To the data of Table 2 can be added the observation that the total growth activity of the two-dimensional chromatogram (Figure 5) was 0.94 g., as compared with 0.73 μ g. for its one-dimensional control.

This has several implications. Firstly, at any spot on a primary chromatogram, the growth activity as assayed does not give an immediate estimate of the total amount of growth-substance potential at that position. In turn, this implies that the substances at each of the three zones are not active in themselves, but that they give rise to growth-active material probably in the mesocotyl or coleoptile segments used for bioassay. In this case the conver-

Table 2. *Comparison of activities (in 10^{-2} μ g., IAA equivalents) of eluates and their sources.*

Zone from which eluate was prepared	Activity	Zones in corresponding eluate, and their activity		
		WP (i)	WP (ii)	WP (iii)
WP (i)	3.8	3.0	6.6	5.3
WP (ii)	6.1	2.0	2.5	5.7
WP (iii)	1.6	—	11.2	7.2

sions at least on the primary chromatogram could not be complete during the assay period. Similar partial conversions might limit the responses on eluate chromatograms and it would be instructive to discover the treatment necessary to liberate the full activity of these precursors. A very slow enzymatic conversion in the assay segments is a possible explanation of these results. It may be that the growth-active material is a product common to all four precursors. Another possible explanation of the increased activity on elution is that there are supra-optimal quantities of auxin present in the primary zones, and that the interconversions involve a dilution, so that the response of the sections increases. This seems unlikely however, since (a) loss during storage would have been expected to give at least an initial rise in apparent activity whereas none was observed (see Table 2) and (b) the peaks on the chromatograms are in the majority of cases symmetrical, i.e. with the greatest response in the central part, where presumably the concentrations are highest; supraoptimal concentrations would have given a twin-peaked spot.

The results of the two dimensional chromatogram show that interconversion can occur in the interval between the two runs; it does not seem to take place during the actual running of the solvent, since it would be expected that the peaks would then show pronounced tailing, (see Housley and Bentley, 1956). The rapidity and apparently spontaneous nature of these interconversions suggest that isomerism or polymerisation may be involved.

In conclusion, the foregoing results emphasize the great difficulties of attaining reliable *quantitative* chromatographic assays of native auxins and stress the need for development of more delicate chemical methods for the precise identification of growth-active spots. The fact that most of the auxin potential in pea roots is not IAA itself but a system of water-soluble auxin precursors present in relatively high concentrations (in fresh extracts probably amounting to more than 10 μ g IAA equivalents per 100 gm. root tissue) has considerable implications for the hormone control of root growth. These points are being actively pursued.

Summary

1. A study has been made of the ethanol soluble growth substances of seedling roots of *Pisum sativum* using paper-partition chromatography and three different biological assays, the oat coleoptile segment test, the oat mesocotyl segment test and the wheat coleoptile segment test
2. To avoid the possible complications due to alkaline conditions, the neutral running solvent *iso*-butanol, methanol, water has been used throughout.
3. Purification of the concentrated extract by the acetonitrile method of Nitsch yielded very variable non-quantitative results. Nevertheless, the following ether-soluble growth-active substances have been shown to be present. *Auxins* at (a) Rf 0—0.15 (possibly identical with "accelerator α " of Bennet-Clark); (b) Rf 0.15—0.3 (possibly IAA); (c) Rf 0.5—0.65 (possibly identical with "Malus 2" of Luckwill and "Ribes 2" of Wright); (d) Rf 0.7—0.9. *Inhibitors* at (a) Rf 0.3—0.5 and (b) Rf 0.7—0.95.
4. The water-soluble, ether-insoluble residue had much greater activity. The active substances could be most easily removed by ethanol. On chromatograms four active growth-promoting spots were identified at Rfs. 0.05 to 0.25; 0.2 to 0.4; 0.45 to 0.65, and 0.75 to 0.95.
5. These substances seem to be spontaneously interconvertible in the solvents used in these experiments, *i.e.* under neutral conditions and in the absence of ammonia. Isomerism or polymerisation is probably involved in these changes.
6. It has not been possible to establish whether these water-soluble substances are indole compounds, whether they are in themselves active auxins or whether they are converted into such in the tissues of the assay segments.

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The Effect of IAA on Root Development of *Acer saccharinum* L

By

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Introduction

There is a well established relation between the presence of leaves or buds and the development of roots on cuttings of a large number of tree species (van der Lek 1934, van Overbeek 1951, Went 1929); and in a previous paper (Richardson 1957), it has been suggested that root development in seedlings may be controlled by the same factors as in the case of cuttings. Experiments with first year seedlings of *Acer saccharinum* demonstrated that during the growing season, the formation of lateral roots is determined by the presence of an active terminal meristem, while root elongation is controlled by a stimulus supplied by the leaves. Since root production in cuttings can often be stimulated by treatment with synthetic auxin (see Pearse 1939, Thimann and Rogers 1950) it was obviously of interest to see if this is also true in the case of *Acer saccharinum* seedlings; and in the present paper two experiments are discussed in which active terminal meristems were replaced by synthetic auxin, and the effects on root development studied.

Experimental methods

Both experiments comprised two series, the first in water cultures and the second in pots containing soil. The seedlings used in both series were germinated and grown as described in previous papers (Richardson 1953, Wassink and Richardson 1951); each had 8 to 10 fully-expanded leaves.

¹ The work described in this paper was carried out at the Laboratory for Plant Physiological Research, Agricultural University, Wageningen, The Netherlands. The paper is the 170th communication of the laboratory.

The nutrient solution used for the water cultures was of the same composition as that in the gravel cultures of previous experiments (Wassink and Richardson 1951). The 1-litre flasks were kept at a temperature of $20^{\circ} \pm 2.0^{\circ}\text{C}$, under illumination from 4 fluorescent tubes (T. L. "Daylight") at an intensity of 4000 lux in the first experiment and 250 lux in the second. The light went on at 0700 hrs. and off at 2300 hrs. From 0700—0900 hrs. and from 2100—2300 hrs. the flasks were aerated with ordinary air by means of a small pump (Austen, Type V/AC), while the nutrient solution was replaced every week. Four first-year seedlings were placed in each flask and transferred to the controlled environment described above 8 days before the various treatments began.

The pots used were ordinary 7" flower pots and were filled with a mixture of 2 parts sandy loam to 1 part leaf mould. One seedling was planted in each pot and transferred to the experimental conditions at the same time as the water cultures. The soil cultures were not, of course, aerated, but were watered daily with tapwater.

Before starting the experimental treatments, all seedlings were removed from the flasks or pots and their roots were pruned with a scalpel so as to leave 8 actively elongating main roots on each plant. The distal 5 cms. of these main roots was cleaned of all lateral roots and the seedlings were returned to their containers. Great care was exercised in removing the seedlings from the soil; they were washed out by means of a gentle stream of water and, as far as could be judged, this treatment had no harmful effects.

The water cultures were then divided into groups for the following treatments: —

1. Control (=untreated).
2. Disbudded, terminal meristem replaced by a smear of lanolin paste.
3. Disbudded, terminal meristem replaced by lanolin and defoliated.
4. Disbudded, terminal meristem replaced by a smear of 0.5 % solution of pure indolyl-acetic acid (IAA) in lanolin.
5. Disbudded, terminal meristem replaced by 0.5 % IAA in lanolin, and defoliated.
6. Disbudded, terminal meristem replaced by a smear of 1.0 % solution of IAA in lanolin.
7. Disbudded, terminal meristem replaced by 1.0 % IAA in lanolin, and defoliated.

There were, thus, 3 levels of IAA application (0.0, 0.5 and 1.0 %) \times 2 seedling treatments ("disbudding" and "disbudding plus defoliation"), together with an untreated control.

The seedlings growing in soil were similarly treated in all respects except that, owing to a shortage of plant material, it was not possible to include the 0.5 % IAA application (treatments 4 and 5 above). In both series, 4 seedlings distributed at random were used for each treatment.

Experiment Results

First experiment

The experimental treatments ran for a period of 3 weeks; the seedlings were then harvested, their roots were cut at the distal end of the region which had been cleaned of lateral roots and all growth during the experiment was recorded.

The results of this experiment are summarised in Tables 1 and 2, and Figures 1 to 5.

Table 1 shows the mean number and length of all new roots produced per treatment in the water cultures, while Table 2 gives these data for the seedlings growing in soil. The last 2 columns in these tables require some explanation. There were treatment differences both in number of new roots produced and in increase in length. Since lateral root formation is obviously a function of main root length, it was clearly desirable, for purposes of comparison, not to rely on number of roots only. In these columns, therefore, the numbers of 1st- and 2nd-order lateral roots counted have been expressed per unit length of, respectively, main roots and first-order laterals.

Photographs of 5 cm.-lengths cut from typical main roots from the various series are pictured in Figures 1 to 5. The "disbudded and defoliated" series is not represented in the photographs since, in both water cultures and soil, the plants died.

Dealing first with the water cultures, Table 1 and Figure 1 demonstrate that, as was expected, the principal effect of disbudding is to decrease the number of new roots formed, as compared with control plants; unexpectedly, however, root elongation was also reduced. It must be remembered, of course, that total root length is a function of the number of roots produced as well as of their length; and when mean root length is considered, the apparent effect of disbudding is considerably lessened. There was considerable variation between seedlings and this difference is barely significant.

When disbudding is accompanied by defoliation there is practically no further root growth and no new roots are produced. At the end of the experiment all the seedlings were dead and the roots had turned a yellow-brown colour.

The two series in which the terminal growing point was replaced by IAA in the presence of leaves may be treated together, and for purposes of assessing the effect of IAA they should be compared with the disbudded plants. Table 1 shows that, in both series, the number of new roots formed increased markedly over the disbudded series. The effect of IAA in increasing the number of 1st-order lateral roots per cm. main root is significant at the 0.1 % level; the difference between IAA levels is significant at the 5 % level.

The main effect of IAA application in the presence of leaves, therefore, was to stimulate root formation with only small and inconsistent effects on root elongation. The differences between 1.0 % IAA and 0.5 % IAA treated plants were considerably less than those between 0.5 % IAA treated and disbudded seedlings.

These effects of IAA application are clearly somewhat modified when the seedlings are first defoliated. The most striking effect of defoliation was to

Table 1. *Acer saccharinum*. Data relating to root production and elongation in water cultures of the first experiment. Means of 4 seedlings.

Treatment	Total growth of main roots (cm)	Total No.	New Roots		Longest root length (cm)	1st Order Laterals			2nd Order Laterals		No. 1st laterals per cm mains	No. 2nd laterals per cm 1st laterals
			Total length (cm)	Mean root length (cm)		Total No.	Total length (cm)	Mean root length (cm)	Total length (cm)	Mean root length (cm)		
Control	44.9	167.2	139.6	0.84	7.6	144.8	117.7	0.81	22.5	21.9	1.73	0.20
Disbudded	34.6	35.0	23.2	0.60	1.5	34.8	23.2	0.60	0.2	0.1	0.48	0.01
Disbudded and defoliated	5.6 ¹	—	—	—	—	—	—	—	—	—	—	—
Disbudded + 0.5 % IAA	40.5	312.5	243.8	0.78	5.4	194.8	199.7	1.04	117.8	44.1	2.50	0.63
Disbudded, + 0.5 % IAA and defoliated	15.5	142.8	44.2	0.32	1.2	142.0	43.8	0.32	0.7	0.4	2.56	0.11
Disbudded + 1.0 % IAA	48.8	439.8	436.8	0.99	5.8	304.2	331.6	1.26	135.5	55.1	3.68	0.35
Disbudded, + 1.0 % IAA and defoliated	16.5	254.0	73.3	0.30	0.8	230.5	70.3	0.31	23.5	3.0	4.10	0.29

¹ Plants dead and roots brown by time of harvest.Table 2. *Acer saccharinum*. Data relating to root production and elongation in seedlings growing in soil in the first experiment. Means of 4 seedlings.

Treatment	Total growth of main roots (cm)	Total No.	New Roots		Longest root length (cm)	1st Order Laterals			2nd Order Laterals		No. 1st laterals per cm mains	No. 2nd laterals per cm 1st laterals
			Total length (cm)	Mean root length (cm)		Total No.	Total length (cm)	Mean root length (cm)	Total length (cm)	Mean root length (cm)		
Control	44.7	198.2	183.3	0.93	4.1	169.8	154.1	0.90	53.5	29.2	2.00	0.32
Disbudded	49.1	53.0	80.4	1.56	3.4	46.0	62.9	1.51	7.0	17.5	0.52	0.10
Disbudded and defoliated	6.9 ¹	—	—	—	—	—	—	—	—	—	—	—
Disbudded + 1.0 % IAA	55.1	503.2	572.7	1.12	7.7	269.8	429.4	1.69	233.5	143.3	2.83	0.46
Disbudded + 1.0 % IAA and defoliated	44.1	430.8	614.9	1.47	4.5	266.8	479.3	1.89	170.0	139.6	3.27	0.36

¹ Plants dead and roots brown by time of harvest.

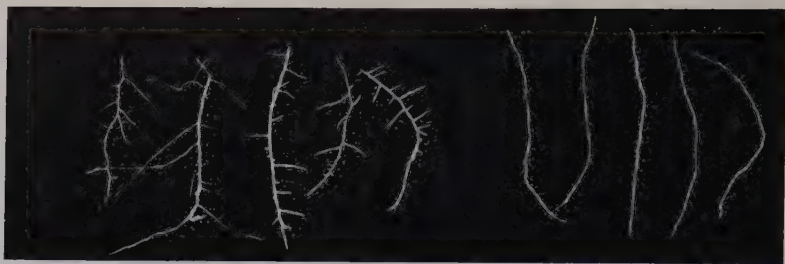


Figure 1. Left: Control, Untreated. Right. Disbudded, with Lanolin.

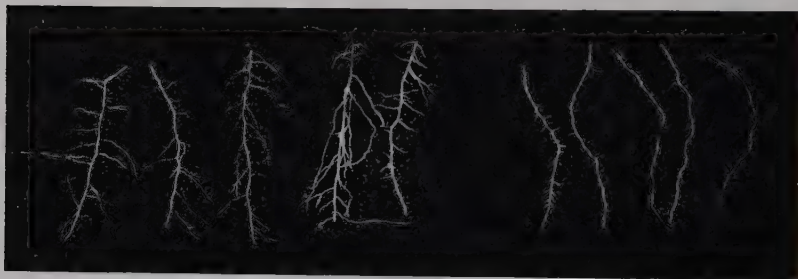


Figure 2. Terminal meristem replaced by 0.5 % IAA. Left. With leaves. Right: Defoliated.

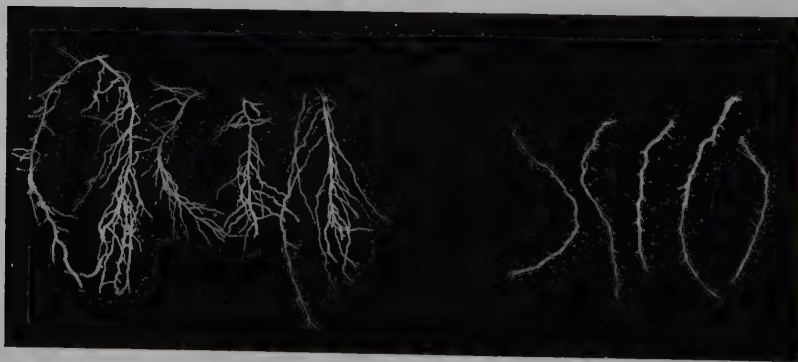


Figure 3. Terminal meristem replaced by 1.0 % IAA. Left: With leaves. Right: Defoliated.

Figures 1 to 3. *Examples of root development in the water culture series of the first experiment. For explanation, see text.*

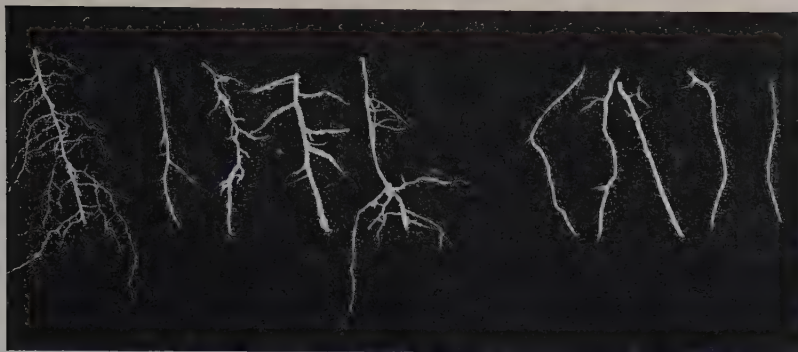


Figure 4. Left: Control, Untreated. Right: Disbudded, with Lanolin.



Figure 5. Terminal meristem replaced by 1.0 % IAA. Upper: With leaves. Lower: Defoliated.

Figures 4 and 5. *Examples of root development in the soil culture series of the first experiment.* For explanation, see text.

inhibit root elongation (Figures 2 and 3), as had been expected from the results described in the previous paper (Richardson 1957).

Thus, most defoliated seedlings had a majority of brown and obviously non-growing roots at the time of harvest. This inhibition to elongation, however, has the effect of masking the influence of IAA on the absolute amount of root formation. For example, the mean total number of roots formed at the 0.5 % IAA level was less than the number formed on the control plants (Column 2 of table 1); the difference, however, was confined to the 2nd-order lateral roots. Since defoliation inhibited elongation of lateral roots, it seems reasonable to ascribe the lack of 2nd-order laterals to an insufficiency of 1st-order laterals on which to grow, rather than to a direct effect of IAA. When relative values are compared, it is clear that, in general, IAA had the same influence as before on root production.

The most significant results of the water culture series may be summarised briefly as follows. Disbudding greatly inhibited root formation while replacement of the terminal meristem by IAA more than overcame the inhibition. Defoliation had no marked effect on root formation, but suppressed growth in length almost completely. These relations can be seen most vividly in Figures 1 to 3. It must be pointed out, however, that, in Figure 3, the photograph of the leafy series gives an exaggerated impression of lateral root length; in fact there was no marked difference in mean root length between the 1.0 % IAA and the 0.5 % IAA series.

Turning now to the series in which seedlings were grown in soil (Table 2 and Figures 4 and 5) it can be seen that roots of the control plants differed only slightly from those in the water cultures; the only significant difference is in the greater number of 2nd-order lateral roots. It is also clear that, as far as the effects of disbudding and the application of IAA in the presence of leaves are concerned, the results were not markedly different from those with the water cultures; thus, disbudding markedly reduced, as compared with the controls, both the absolute number of 1st- and 2nd-order lateral roots formed and the amount relative to length of main and 1st-order lateral roots. These reductions are of the same order as in the water culture series. There was no change in main root growth and, as before, the total length of both 1st- and 2nd-order lateral roots was reduced. Mean root length, on the other hand, was greater on disbudded plants than on the controls but this difference is not significant.

The application of 1.0 % IAA in lanolin had about the same effect on seedlings growing in soil as in water cultures. As before, there was an apparent stimulating effect of IAA on lateral root growth in length, but again, this effect is due largely to the influence of IAA on root formation and not upon elongation. When mean root lengths are compared there are

no significant differences between treatments. It may be concluded, therefore, that, as in the water cultures, IAA application in the presence of leaves increased root formation, but had no significant influence on elongation.

The series which was defoliated before the application of IAA shows the most striking difference between soil and water cultures. In the water cultures, defoliation had no marked effect on root formation but it suppressed elongation almost completely. In the plants growing in soil, the lack of any significant effect of defoliation on root formation was confirmed but there was, further, no significant effect of defoliation on root elongation. Thus, there was no significant difference in total root length between defoliated and leafy plants, while, on the basis of mean root length, the defoliated seedlings showed a slightly higher value than the plants with leaves.

It appears from this experiment that IAA in a lanolin paste can replace the terminal meristem as the controlling influence in root formation, but it cannot replace the leaves in supplying the stimulus necessary for root elongation. The role of the leaves in supplying this latter factor, however, can be obviated by growth in a humus-rich soil.

Second experiment

In view of the unexpected influence of the growth medium on root elongation observed in the first experiment, a repetition seemed desirable. It was decided to repeat the experiment at a light intensity of 250 lux instead of 4000 lux for the following reason. In the experiment at 4000 lux, one of the effects of defoliation was to reduce photosynthesis. Quite apart from any hormonal influence of the leaves on root elongation, it is to be expected that defoliation will result in an immediate decrease in the supply of carbohydrates to the roots. In this experiment, therefore, differences in root elongation between defoliated and leafy plants may well have been exaggerated by differences in level of photosynthesis; by performing the experiment at 250 lux such differences could be minimised.

The results of this experiment will not be presented in detail since they are qualitatively similar to those of the first experiment. The only striking effect of the lower light intensity was to reduce the absolute amount of root growth. The total number of roots formed in all treatments, and the total length of new roots in all treatments except the 1.0 % IAA series were reduced by about 30 %. In the 1.0 % IAA series, root elongation was reduced by over 50 %.

Apart from this effect, the influence of treatment was essentially the same as in the first experiment.

Discussion

The experiments discussed in this paper can be regarded as preliminary only and the results must be treated with some caution. In the first place, the experimental methods were not ideal, especially with regard to the seedlings grown in soil. It is not to be expected that removal from the soil, drastic root pruning and replacement will be without any effect on root growth, although in both experiments no great differences in growth between seedlings in water culture and those in soil could be detected.

Another limitation in experimental method lies in the measurement of root formation. In assessing root production, visible roots only were counted, no allowance being made for root initials which did not elongate. In defoliated seedlings, where root elongation was inhibited, this number may have been considerable; and this might explain the apparent inhibiting effect of defoliation on root formation in these experiments. It may well be that defoliation had no influence on the formation of root initials and that the apparent effect was an illusion due to counting visible roots only.

In spite of these limitations, however, some interesting features emerge from the results. Dealing first with the water culture series, it may be concluded that in so far as the effects of disbudding and defoliation are concerned, previous conclusions have been confirmed. Thus, in both experiments, disbudding inhibited root formation but had no significant effect on elongation of roots already present; defoliation, on the other hand, had no real effect on root formation, but suppressed root elongation. Replacement of the terminal meristem by a solution of IAA in lanolin more than overcame the effect of disbudding on root formation, but was unable to replace the leaves in supplying the stimulus necessary for root elongation. The fact that the results of the experiment at 250 lux were qualitatively the same as those observed at 4000 lux indicates that the effect of defoliation was not due to a deficiency of carbohydrates.

These findings are straight-forward and were not unexpected. The soil cultures, however, gave surprising results. From the evidence presented here, there can be little doubt that the normal rôle of the leaves in stimulating root elongation is not always an essential one; with seedlings growing in a humus-rich soil, under the conditions of the present experiments, normal root elongation is possible in the absence of leaves. Assuming that root elongation in the soil cultures was not a traumatic effect due to the washing out of the roots, it seems that the soil supplied a growth-promoting stimulus normally provided by the leaves and not provided by the nutrient solution.

In this connection, a paper by Hitchcock and Zimmerman (1952), is of interest. They record that indolylbutyric acid is only effective in stimulating

root development in apple cuttings when they are planted in peat; in moist sand, no roots develop. Although the authors conclude that the role of the peat is in root formation, it may equally well stimulate elongation. Similarly, Fowells (1943) found that IAA only stimulates root production in seedlings of *Pinus ponderosa* when they are grown in soil; in water culture no effect was recorded.

As to the nature of the elongation stimulus, the evidence of previous workers suggests that it might be a vitamin. Numerous investigators have found that vitamins of the B group can stimulate root elongation on cuttings without affecting root formation (Chadwick and Swartley 1940, Stoutemyer 1940, Warner and Went 1939, Went *et al.* 1938). Furthermore, there is abundant evidence that vitamins play a role in elongation of excised roots. Apparently unlimited growth of excised roots was first claimed by White (1934), using a solution of mineral salts, sugar and dried yeast. The necessity for a yeast extract was confirmed by Robbins and Schmidt (1938), who found that the essential factor was vitamin B₁. Since then, many investigators have demonstrated that vitamins play an essential role in root elongation (see *e.g.* Robbins 1951).

Of course, demonstrating that vitamins are essential for root development does not prove that, in the present experiments, the growth factor under discussion is a vitamin. Nevertheless, such a contention would not be contradicted by the finding that a humus-rich soil can replace the leaves as a source of the growth factor. Vitamins of the B group are known to be produced by biological activity in soil and they have been extracted from decaying plant material (see Harley 1948); Mc Dougal and Dufrenoy (1940), consider that vitamins can be taken up by tree roots, via the agency of mycorrhizal fungi. On present evidence, however, further speculation would not be justified.

Summary

Experiments are described in which the terminal meristems of first-year seedlings of *Acer saccharinum* growing in water cultures were replaced by artificial auxin (indolylacetic acid) in lanolin. At concentrations of 0.5 and 1.0 %, auxin more than overcame the inhibiting effect of removing the terminal meristem on root formation. The roots only elongated, however, on leafy seedlings; on defoliated plants root elongation was suppressed.

When seedlings growing in a humus-rich soil were used, root elongation was normal, irrespective of whether or not leaves were present.

There were no qualitative treatment differences between seedlings growing at a light intensity of 4000 lux and plants growing at 250 lux, indicating that the effect of defoliation was not due to a deficiency of carbohydrates.

It could be concluded, therefore, that apart from photosynthesis, root development is determined by at least two accessory factors. During the growing season one is elaborated in the terminal meristem and controls root *formation*; its action can be replaced by artificial auxin. The other is formed in the leaves and determines root *elongation*; in the absence of leaves, it can be supplied by a humus-rich soil. The evidence of literature suggests that the latter factor may be one of the B vitamins.

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A Study on the Relationship between Water Uptake and Respiration of Isolated Bean Germ-axes

By

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The present author has reported that the water uptake process of the isolated bean germ-axes contains three distinctly resolvable steps, *i.e.*, the initial steep rise (Phase A, perhaps uptake by diffusion) and the final steady ascent of aerobic nature (Phase C) after the transient pause of uptake (Phase B) (Oota 1957). The results described below suggest that the function of the cytochrome *c*/cytochrome oxidase system may be associated with the aerobic phase of water uptake (Phase C) directly or indirectly.

Materials and Methods

Seeds of *Vigna sesquipedalis* stored in a dark desiccator for about a year after harvest were used. Similar methods with slight modifications were applied to those described elsewhere of obtaining the germ-axes (hypocotyls+radicles) of constant humidity and of assaying gaseous exchange, water uptake, total sugar (Oota 1957), and total and protein nitrogens (Izawa 1958). In each respirometer flask were placed 10 germ-axes (the initial weight=18.0 mg.) in the main room and 1.0 ml. of poison solution buffered with phosphate mixture (pH 6.0, the final concentration=1/150 *M*) in the side arm. The incubation was continued routinely for 4 hrs. The first reading of respirometers was made after 15 min. of the liquid addition to the tissues by the reason stated elsewhere (Oota 1957). When the action of a given poison on Phases C and II was studied, the tissues were previously shaken with 0.5 ml. of phosphate buffer (pH 6.0, 1/150 *M*) for 2 hrs. before 0.5 ml. of the buffered poison solution was supplemented from the side arm. It is known that

oxygen uptake of the imbibing tissues increases exponentially (Phase I, $R.Q.=1.0$) until it reaches a linear elevation (Phase II, $R.Q. > 1.0$); the time of the shift from Phase I to Phase II nearly agrees with that from Phase B to Phase C of water uptake (Oota 1957). Phases A, B and I were found to be passed normally in this previous incubation period. For cyanide experiments, a KOH-KCN mixture as a CO_2 absorbent was used. To examine the effects of KCN on Phases C and II the respirometers were temporarily withdrawn from the bath after the previous 2 hr. incubation and KOH-KCN and KCN-phosphate solutions were pipetted into the inner cups and the side arms respectively. The procedure took 10 min. KCN was added to the tissues after further 10 min. of temperature equilibration, and the respirometers were shaken for subsequent 2 hrs. The pH of the medium was shifted up to pH 6.4 by the addition of 10^{-3} M KCN. Sodium diethyldithiocarbamate (DIECA) was examined at pH 7.0 (cf. Jostein). In the control runs, buffered poison solutions were replaced with pure phosphate mixture (1/150 M) of respective pHs.

In the cytological examinations was used a combination of Carnoy-Feulgen which permitted the most satisfactory observation of nuclei out of various combinations examined of fixatives (Carnoy, Telyesniczky and 5 per cent trichloroacetic acid) and dyes (Feulgen and hematoxylin). The germ-axes were shaken in the bath for 7 hrs. and several axes for each time were withdrawn at regular intervals of 30 min. for the first 2.5 hrs. and of 60 min. thenceforth, to be fixed, prepared into paraffin sections and stained.

The cotyledons were isolated from dry seeds, decoated and stored in a desiccator over silica-gel for several days until constant weight was attained before use. Each respirometer flask contained 5 cotyledons (the initial weight=ca. 250 mg.) and 1.5 ml. phosphate buffer (pH 6.0, 1/150 M).

The respirometers were shaken at $30^\circ C$. in the dark. All estimations were repeated on at least two separate occasions, the mean values being shown below.

Results and Discussion

Cytological observations. Goo's report on water uptake of intact coniferous seeds appears to suggest a possibility of the occurrence of cell division in the time course of water uptake, in particular in Phase C, of the present materials (cf. Oota 1957). No change, however, occurred in morphology of nuclei in any part of the germ-axes during the 7 hr. incubation. No mitotic figure was found except that most nuclei in the root tip meristem were maintained apparently at early prophase throughout the period examined.

Nitrogen change in the imbibing tissues was investigated (Figure 1). Change in content of neither protein nor total nitrogen in the tissues took place during the 4 hr. incubation. That a negligible amount of nitrogen appeared in the medium in the first 30 min. period and maintained constant thereafter would merely mean that some nitrogen compounds attached on the tissue surface were liberated into the medium. It will, therefore, be deduced that in the present materials water uptake in association with *growth* is likely excluded.

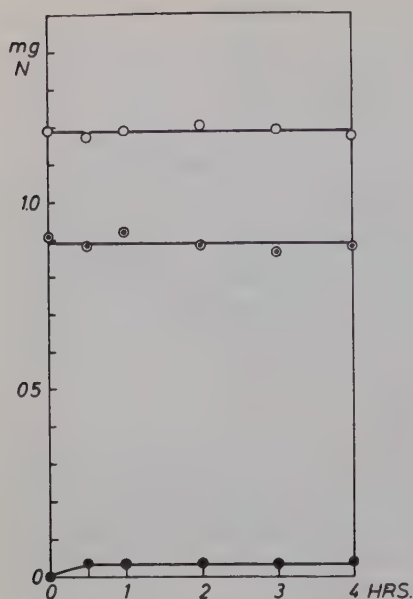


Figure 1. Nitrogen change in the imbibing bean germ-axes. Warburg respirometers used; air, 30°C., darkness. Phosphate buffer (pH 6.0, 1/150 *M*) added at zero time to the desiccator-dried tissues. On the abscissa time in hours, on the ordinate mg. N per 18 mg. initial fresh weight, ○—○ total N in tissues, ⊙—⊙ protein-N in tissues, ●—● total N in medium. For further details see the text.

No cell division and probably no nitrogen change is involved, and the metabolic activities of the tissues are likely restricted to the carbohydrate change, *i.e.*, respiratory and fermentatory breakdown, exudation and starch synthesis, detailed accounts of which have been given in the previous paper (Oota 1957).

Sugar exudation. A striking parallel has been noticed between the water uptake and the sugar exudation (Oota, 1957). The former, however, was found to be practically independent of the latter. The results are not detailed here, but the tissues could absorb water nearly normally even when they were shaken in a medium containing enough sugar (1/25 *M* sucrose) to render the sugar exudation negligible. The sugar exudation, therefore, will be carried out by diffusion; the absorbed water may control the mobility of sugar in the tissues, which in turn may limit the exudation in question.

Effects of poisons. The effects of a variety of metabolic poisons on the processes of water uptake and gaseous exchange are illustrated in Figures 2 to 9 and summarized in Table 1.

Of the metal reagents used, KCN (examined at 10^{-3} *M*) and NaN_3 (10^{-3} *M*) are known to combine with both iron and copper, while α, α' -dipyridyl (2×10^{-3} *M*) and *o*-phenanthroline (2×10^{-3} *M*) rather specifically with iron, and DIECA (2×10^{-3} *M*) and salicylaloxime (2×10^{-3} *M*) with copper. Phase A was resistant to these metal reagents, whereas they could produce appreciable depression of Phase C. As regards oxygen consumption, KCN and NaN_3

Figure 2. *Effects of KCN (10^{-3} M) on water uptake and gaseous exchange of the bean germ-axes.* Warburg respirometers used; air, $30^{\circ}\text{C}.$, darkness, pH 6.4. A: Poison added at time zero to the desiccator-dried tissues. B: Poison added at 2 hrs. of water absorption. On the abscissa time in hours, on the ordinates $\mu\text{l. gas}$ per 18 mg. initial fresh weight (left), mg. fresh weight per 18 mg. initial fresh weight (right), —○—: O_2 uptake, —●—: CO_2 output, —+—: fresh weight,: controls (1=fresh weight, 2= CO_2 , 3= O_2). For further details see the text.

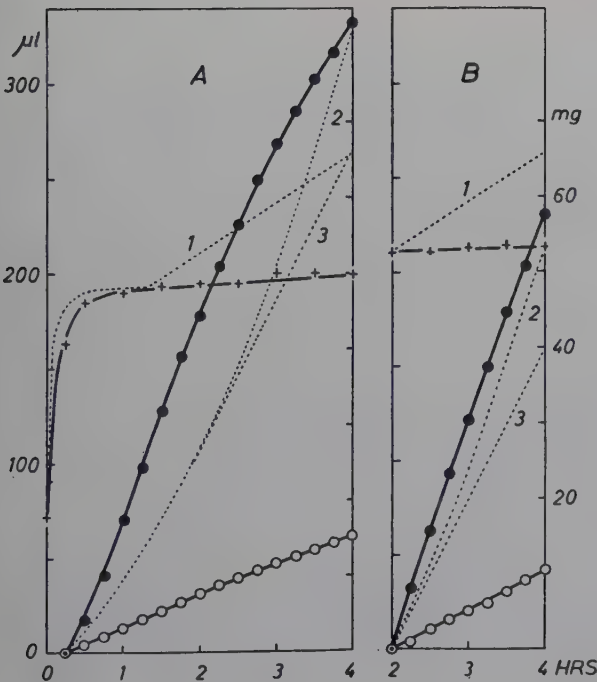
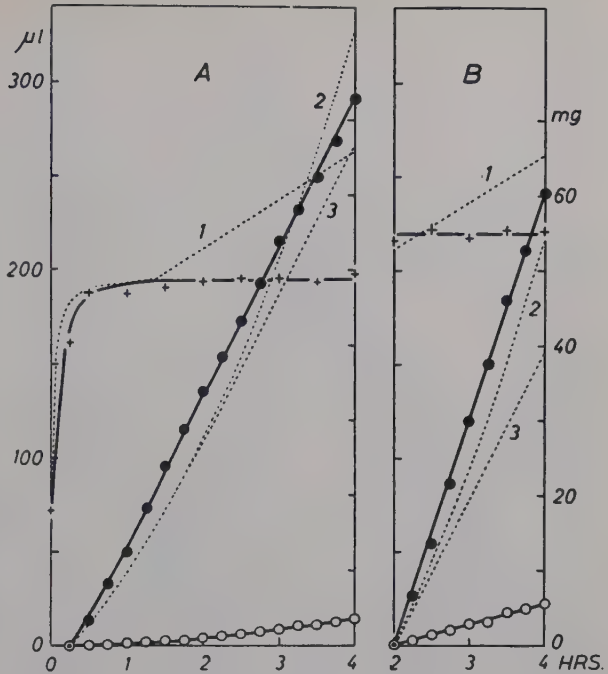


Figure 3. *Effects of NaN_3 (10^{-3} M) on water uptake and gaseous exchange of the bean germ-axes.* Warburg respirometers used; air, $30^{\circ}\text{C}.$, darkness, pH 6.0. For further details see the explanation of Figure 2.

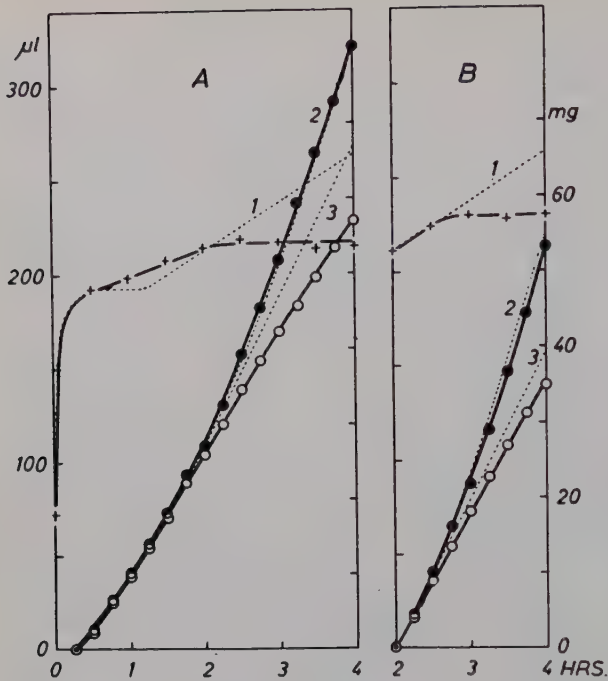


Figure 4. Effects of α, α' -dipyridyl ($2 \times 10^{-3} M$) on water uptake and gaseous exchange of the bean germ-axes. Warburg respirometers used; air, $30^\circ C.$, darkness, pH 6.0. For further details see the explanation of Figure 2.

reduced greatly both Phases I and II. The copper-specific reagents caused an inhibition (more than 20 per cent) only of Phase I, and were entirely inefficacious to Phase II. In contrast with this, the iron-specific reagents diminished Phase II (by ca. 10 to 20 per cent) but not Phase I. Exceptionally phenanthroline could also suppress Phase I. The tissues were conspicuously pink-coloured within 30 min. after the addition of not only dipyridyl but phenanthroline, indicating ready entry of these reagents and the presence of ferrous iron in the tissues. It is, however, known that phenanthroline can react also with minute amounts of copper (cf. Sandell p. 363), and the inhibition of Phase I by this reagent will not inevitably be attributed to the participation of iron in the respiration in question. The above change in sensitivity of the respiratory activity to metal reagents seems to suggest a rather rapid shift in the respiration pattern of the imbibing germ-axes, *i.e.*, a change from respiration via copper enzyme to that via iron enzyme. With all likelihood the iron respiration may be mediated by the cytochrome c/cytochrome oxidase system (cf. Oota 1955). From the very beginning of the incubation, however, the germ-axes were found to give positive Nadi reaction. The mechanism of this shift, if it occurs, in respiration pattern is yet obscure.

Figure 5. *Effects of o-phenanthroline (2×10^{-3} M) on water uptake and gaseous exchange of the bean germ-axes. Warburg respirometers used; air, 30°C ., darkness, pH 6.0. For further details see the explanation of Figure 2.*

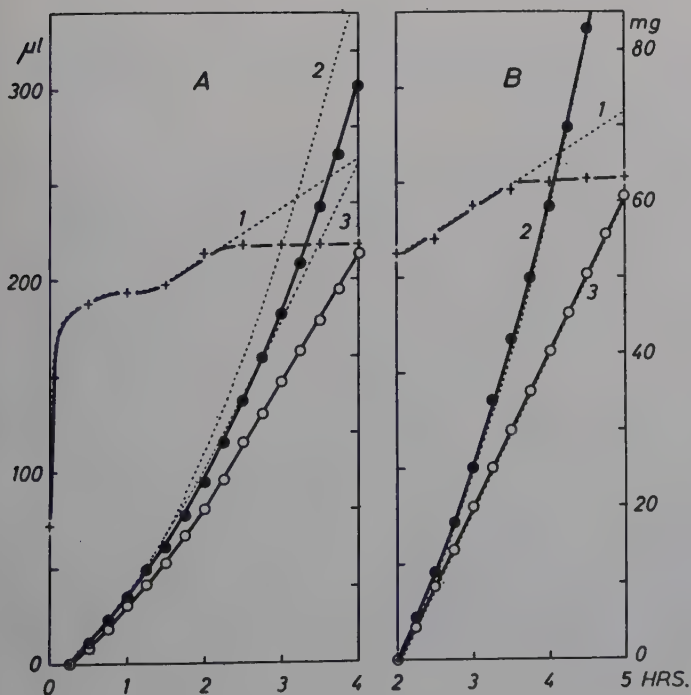
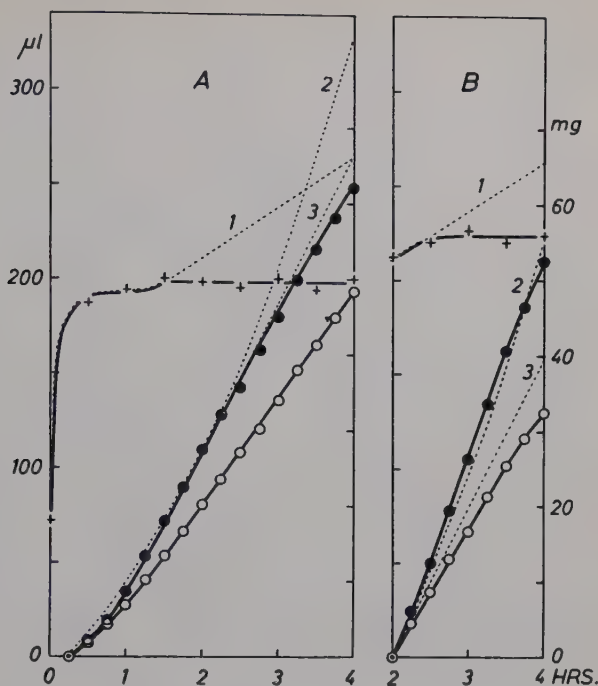


Figure 6. *Effects of sodium diethyldithiocarbamate (2×10^{-3} M) on water uptake and gaseous exchange of the bean germ-axes. Warburg respirometers used; air, 30°C ., darkness, pH 7.0. For further details see the explanation of Figure 2.*

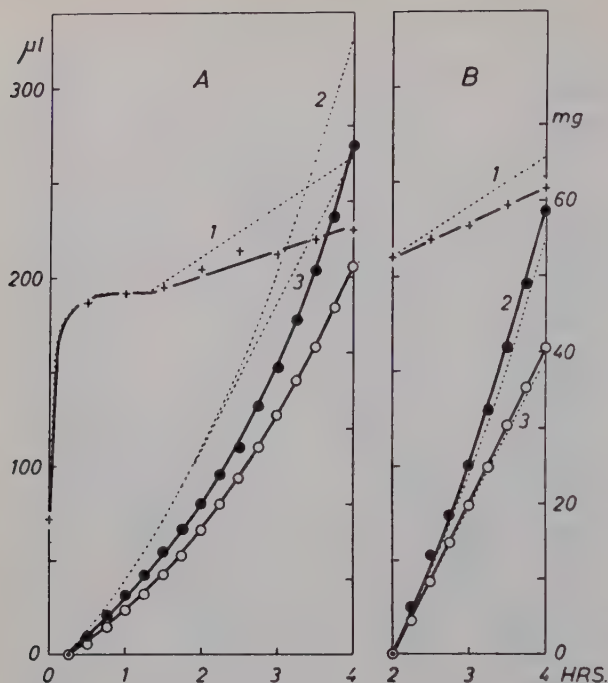


Figure 7. Effects of salicylaldoxime (2×10^{-3} M) on water uptake and gaseous exchange of the bean germ-axes. Warburg respirometers used; air, 30°C ., darkness, pH 6.0. For further details see the explanation of Figure 2.

A possibility will be that the substrate/dehydrogenase system(s) which can link with the cytochrome c/cytochrome oxidase system must be established before the latter will actually function.

It is striking that every metal reagents examined exerted very much greater inhibitory action on Phase C than on Phase II. The copper reagents gave the extreme case, reducing Phase C markedly without any inhibition of Phase II. Moreover, in the experiments with dipyrldyl, phenanthroline and DIECA, the depression of Phase C began with a lag period of 0.5 to 1.5 hrs. The lag period was evidently shorter when the poison was added at zero time than was when added at 2 hrs. of incubation. No such lag was found for the gaseous metabolism. These facts imply that the primary effect of the reagents is an inhibition of general protoplasmic activities of aerobic nature, including naturally the iron respiration, and thereby the Phase C water uptake. According to Hackett and Thimann, in potato tissues, too, water uptake is more sensitive than respiration to various respiration inhibitors. They have concluded that the water uptake of the tissues is not geared to the total but to a small fraction of respiratory activity. In their long term experiments, however, it is not improbable that *growth* of cells occurs (cf. Carlier and Buffel).

Figure 8. *Effects of disodium arsenate ($2 \times 10^{-3} M$) on water uptake and gaseous exchange of the bean germ-axes. Warburg respirometers used; air, $30^{\circ}C.$, darkness, pH 6.0. For further details see the explanation of Figure 2.*

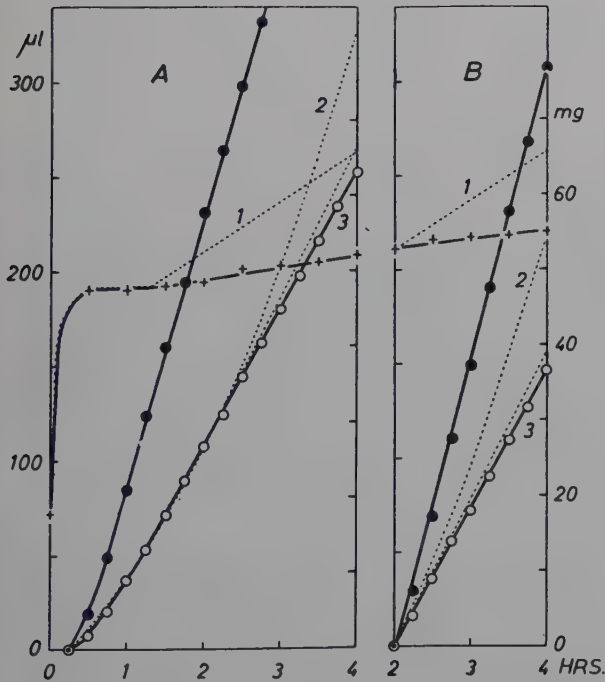
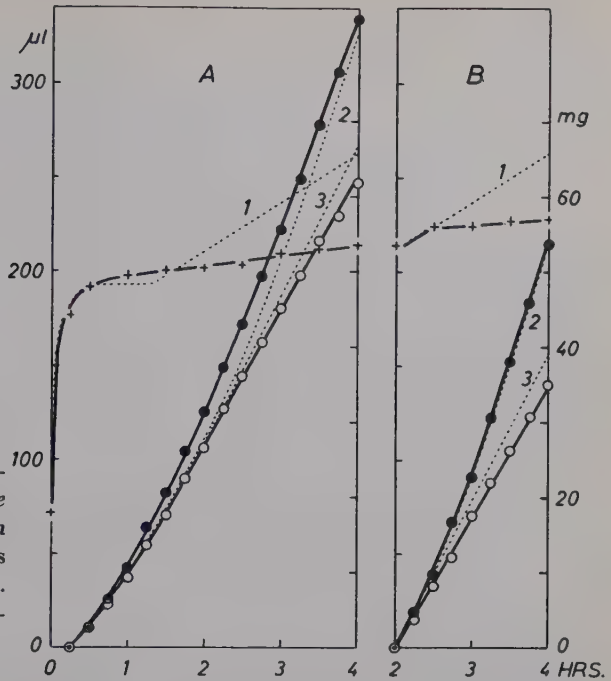


Figure 9. *Effects of 2,4-dinitrophenol ($10^{-4} M$) on water uptake and gaseous exchange of the bean germ-axes. Warburg respirometers used; air, $30^{\circ}C.$, darkness, pH 6.0. For further details see the explanation of Figure 2.*

Table 1. *Effects of metabolic poisons on various phases of water uptake and gaseous exchange of the isolated bean germ-axes.* The values given referred to inhibition (—) or acceleration (+) in per cent of the controls without poison; computation based on the results shown in Figures 2 to 8.

Poison	Poison added at $t = 0'$			Poison added at $t = 120'$		
	Phase A ¹	Phase I ²		Phase C ³	Phase II ³	
		O ₂ -uptake	CO ₂ -output		O ₂ -uptake	CO ₂ -output
KCN ($10^{-3}M$)	0	— 96	+ 33	— 100	— 87	0
NaN ₃ ($10^{-3}M$)	0	— 66	+ 82	— 100	— 74	— 4
α , α' -Dipyridyl ($2 \times 10^{-3}M$)	+4	0	0	— 100	— 13	0
<i>o</i> -Phenanthroline ($2 \times 10^{-3}M$)	0	— 23	— 2	— 100	— 23	— 16
DIECA ($2 \times 10^{-3}M$)	0	— 18	— 10	— 48	0	0
Salicylaldoxime ($2 \times 10^{-3}M$)	0	— 38	— 22	— 33	+ 3	+ 10
Arsenate ($2 \times 10^{-3}M$)	+4	0	+ 15	— 85	— 13	0
2, 4-Dinitrophenol ($10^{-4}M$)	0	0	+ 120	— 82	— 8	+ 30

¹ Effects on water uptake in the 0'—60' period are shown.

² Effects on gaseous exchange in the 15'—75' period are shown.

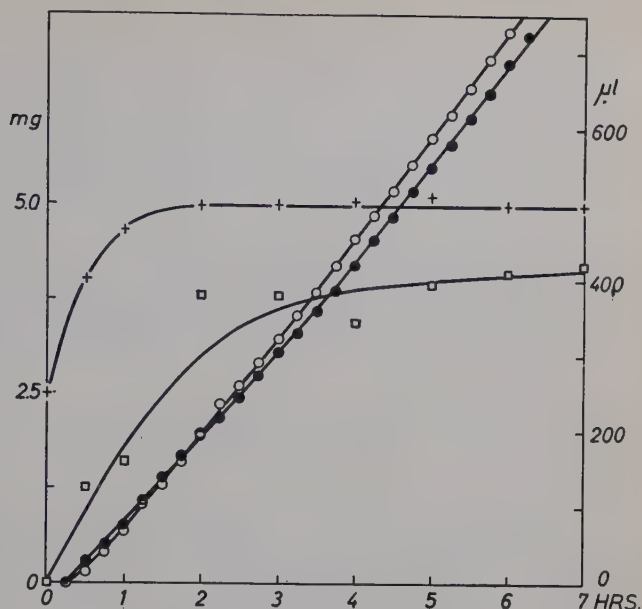
³ Effects on water uptake or gaseous exchange in the 180'—240' period are shown.

The uncouplers examined, i.e., disodium arsenate ($2 \times 10^{-3} M$) and 2,4-dinitrophenol ($10^{-4} M$), had no effect on the oxygen uptake in Phase I and produced a slight inhibition (amounting to ca. 10 per cent) of that in Phase II, while they caused strong inhibition (more than 80 per cent) of Phase C. These results suggest that energy-rich phosphate produced by aerobic exergonic reaction may drive the Phase C water uptake directly or indirectly. Phase A was also entirely resistant to these reagents.

The respiratory quotient of the germ-axes begins to surpass unity as the tissues come into Phase II (Oota 1957). But no simple relationship was seen between carbon dioxide production and water uptake in the presence of poisons (Table 1). It is, however, worth noting that the uncouplers, KCN and NaN₃, all being wellknown inhibitors of the Pasteur effect, were able to provoke intense stimulation on the carbon dioxide production in Phase I and gave only small or even no effect on that in Phase II. This appears to be of importance because here rather rapid decrease of the capability of the imbibing germ tissues of manifesting the Pasteur effect is likely deduced. The view is in agreement with the above cited fact that in Phase II gradually growing aerobic fermentation (a drop of Meyerhof quotient, Oota 1957) occurs.

Water uptake of isolated cotyledons. In view of a specific respiration pattern of the bean cotyledons it must be interesting to see the water uptake process of the reservoir tissues. It has been revealed that at the early germination stage the cotyledons contain cytochromes a and b, but not the

Figure 10. Water uptake, gaseous exchange and sugar exudation of the isolated bean cotyledons. Warburg respirometers used; air, 30°C., darkness. Phosphate buffer (pH 6.0, 1/150) *M* added at zero time to the desiccator-dried tissues. The sugar values referred to the amounts of total sugar in the medium. On the abscissa time in hours, on the ordinates 100 mg. fresh weight and mg. hexose per 250 mg. initial fresh weight (left), μ l. gas per 250 mg. initial fresh weight (right). +—+ fresh weight, \square — \square hexose, \bullet — \bullet CO_2 , \circ — \circ O_2 .



c component (Kumada); nitrate reductase is present which can function as a terminal oxidase (Kumada, Egami *et al.*). Relevantly TCA cycle is unlikely operative in the cotyledons (Oota *et al.* 1953). Figure 10 illustrates the time course of water uptake together with those of gaseous exchange and sugar (=total sugar) exudation of the cotyledons shaken in a phosphate buffer under air. As shown, the initial rapid uptake of water (comparable to Phase A, and may be carried out by diffusion) is followed by no such steady rise in uptake as that of Phase C in a prolonged (7 hr.) incubation. The situation is strikingly similar to that of the germ-axes under nitrogen (Oota, 1957), and seems to be in favour of a hypothesis that the typical aerobic pattern of respiration (the cytochrome c/cytochrome oxidase system) would be a prerequisite to the occurrence of the aerobic water uptake such as that of Phase C.

The exact nature of the linkage between water uptake and respiration, if it really exists in the germ-axes, remains to be clarified. The above stated uncoupler experiments suggest an energetic correlation, but a preliminary attempt to promote Phase C by the addition of 2×10^{-3} *M* ATP together with equimolar MgCl_2 has been failed; oxygen consumption was not affected at all, and Phase C was even depressed by as much as ca. 45 per cent.

In passing, in Figure 10, rather sluggish exudation of sugar is noticed in comparison with relatively steep rise of fresh weight at the outset of in-

cubation, and steady phase of sugar exudation similar to Phase C' which takes place in the germ-axes concomitantly with the Phase C water uptake (Oota 1957) is scarcely discernible. These have also been the case for the imbibing germ-axes under anaerobic condition (Oota 1957). The final concentration of sugar in the medium is roughly computed to be at most $1/150 M$ which may not be the cause of the absence of the Phase C-like step in the water uptake process of the cotyledons. Although nitrogen change was not investigated, the initial levels of total as well as protein nitrogens are expected to be maintained unaltered in the cotyledons during the whole incubation period just as was the case for the imbibing germ-axes (cf. Oota *et al.* 1956).

Summary

The effects of metal reagents, KCN, NaN_3 , α, α' -dipyridyl, *o*-phenanthroline, diethyldithiocarbamate (DIECA) and salicylaldehyde, and uncouplers, arsenate and 2,4 -dinitrophenol, on various phases of water uptake and gaseous exchange of the isolated bean germ-axes have been investigated. The water uptake in Phase A was little affected by all the reagents examined, while that in Phase C was reduced markedly by them. The oxygen uptake in Phase I was not affected by the uncouplers but inhibited by the metal reagents excepting dipyridyl. The oxygen uptake in Phase II was inhibited by the reagents other than salicylaldehyde and DIECA. A lag period was found in the action of dipyridyl, phenanthroline and DIECA. The carbon dioxide production, particularly that in Phase I, was promoted by the uncouplers, KCN and NaN_3 . Any indication of neither cell division nor the nitrogen change was obtained in the imbibing germ-axes.

The isolated cotyledons absorbed water likely only by diffusion for relatively short time, the aerobic phase of water uptake comparable to Phase C of the germ-axes being absent.

Physiological meanings of these and other relevant findings were discussed.

The author is indebted greatly to Mr. Takahisa Ôta of this institute for the cytological examinations.

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The Redistribution of Radioactivity in Geotropically Stimulated Plants Pretreated with Radioactive Indoleacetic Acid

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Introduction

Any tropic bending of plant organ is attributed to an unequal growth between concave and convex sides of the curvature, and the unequal growth might logically be explained by an asymmetric concentration of auxin(s) in the tissue. The theory concerning the mechanism of such asymmetry in a geotropically stimulated organ, as present research extends, is trichotomic. One, postulated by Cholodny and Went (Went and Thimann 1937), is that gravity brings about an unequal distribution of endogeneous auxin. This was substantiated later by two pertinent experimental findings: (a) Lundegårdh (1942) showed that the roots of peas and corn exhibited a differential electric potential on upper and lower sides following geotropic stimulation, and (b) that a differential electric potential installed on two sides of *Avena* coleoptiles caused lateral transport of indoleacetic acid and curvature (Webster and Schrank, 1953). The second, speculated by Audus and Brownbridge (1957), is that geotropic stimulation causes *de novo* production of an endogeneous growth inhibitor on the lower side of the root, thus the initial curvature occurs. The third, proposed recently by Rufelt (1957), is that geo-

electric reaction increases auxin production on the lower side of a horizontally placed plant. While the last two hypotheses still await a demonstration of direct geotropic induction of auxin- or inhibitor-production, the first theory could be tested by tracer technique. Thus, the present study was initiated.

If endogeneous auxin is redistributed by gravity or geoelectric force, then it seems probable that absorbed exogeneous auxin should be influenced similarly. Indoleacetic acid is known as a natural auxin of wide occurrence in plants, thus it was used in these experiments to determine whether a redistribution is possible in geotropically stimulated organ. In these experiments, carboxyl carbon labelled indoleacetic acid (IAA-1-C¹⁴) was administered by root or shoot absorption *in vivo* before stimulation.

Materials and Methods

The IAA-1-C¹⁴ with a specific activity of 9.12×10^5 cpm/mg. was used. Plant materials studied were young seedlings of Alaska pea (*Pisum sativum* var. *Alaska*), hybrid corn (*Zea mays*, W23 X oh 5 la) and lima bean (*Phaseolus lunatus* var. *macocarpus*). Roots or shoots of intact seedlings with uniform size (2—3 cm. in length) were pretreated for various lengths of time in 10^{-5} M IAA-1-C¹⁴ solution with 0.005 M phosphate buffer at pH 5.2. At the end of the prescribed pretreatment time, the roots or shoots were rinsed several times in distilled water. The total absorption after pretreatments was determined in the first experiment by counting the radioactivity of ethyl alcohol extract of treated roots with a thin mica end window G-M tube. For the determination of redistribution, the treated plants were mounted on a plastic holder and incubated or stimulated horizontally in a moist chamber with a relative humidity of 95 % for various lengths of time. After the stimulation, root tips of 6—8 mm. in length were cut into upper and lower halves from which fresh weights were taken. The differences between fresh weight of upper and lower halves were found to be less than 10 % of their total weight in all the experiments. In the first experiment, each half was dried under an infrared lamp and the radioactivity was counted directly from the dried tissue. In the experiments conducted later, IAA-1-C¹⁴ was quickly extracted with 95 % ethyl alcohol from each homogenized half. Duplicate aliquots of this ethyl alcohol extract were dried in cupped planchets under an infrared lamp, then the radioactivity was counted. The residue was found to contain approximately 2—10 % of the total when pretreatment time was less than 4 hours thus this fraction was disregarded in the calculation of results. In the experiment number 8, corn seedlings were decapitated 1.5—2 mm. from the root tip before pretreatment in order to eliminate the interference of endogeneous auxin in the possible gravity induced lateral transport of auxin. All experiments were conducted under dim light at room temperature.

Results

The results of all the experiments are summarized in Table 1. The total absorption of IAA-1-C¹⁴ and its subsequent redistribution in geotropically stimulated seedlings were determined as counts per minute per mg. of dry

Table 1. *Absorption of IAA-1-C¹⁴ and redistribution of radioactivity in geotropically stimulated seedlings.*

Exp. no.	Material and no. of seedlings	Pretreatment		Duration of geotropic stimulation (min.)	Radioactivity			
		duration (min.)	total absorption (cpm/mg. DW)		cpm/mg. DW		% distribution	
					upper	lower	upper	lower
1	pea roots 9 each	60	16.0	30	9.4	12.1	43.72	56.28
		120	14.2 ¹	60	7.2	5.9	54.96	45.04
		180	20.8	45	12.2	12.2	50.00	50.00
		270	27.6	90 ²	18.6	22.2	45.58	54.42
		330	34.9	45	31.3	33.9	48.00	52.00
2	pea roots 27 each	60	—	45	35.8	35.3	50.35	49.65
		60	—	90 ²	23.2	25.6	47.54	52.46
3	pea roots 18 each	120	—	90	17.0	19.3	46.83	53.17
		120	—	135 ²	18.8	20.5	47.83	52.17
		120	—	180 ³	15.6	20.0	43.82	56.18
4	pea roots 29 each	210	—	45	50.8	52.5	49.17	50.83
		210	—	90	42.8	45.0	48.75	51.25
5	Lima bean roots 7 each	120	—	60	62.6	71.6	46.64	53.36
		120	—	90 ²	65.8	52.2	55.76	44.24
		120	—	150 ³	58.8	48.0	55.05	44.95
		120	—	180 ⁴	51.0	52.6	49.22	50.77
6	Lima bean roots 8 each	120	—	45	36.6	45.8	44.41	55.59
		120	—	90 ²	37.2	43.8	45.92	54.08
		120	—	135 ³	46.4	48.6	48.84	51.16
7	Corn roots 24 each	120	—	45	51.2	55.0	48.21	51.79
		120	—	90 ²	50.4	56.0	47.36	52.64
		120	—	150 ³	42.0	44.3	48.66	51.34
8	decapitated corn roots 10 each	60	—	45	19.7	20.9	48.52	51.48
		60	—	90	21.6	26.6	44.81	55.19
		60	—	135	22.9	33.3	40.74	59.26
9	pea shoots 16 each	60	—	60	8.2	8.3	49.69	50.31
Average							48.09	51.91

¹ The starting activity of IAA-1-C¹⁴ solution was lower than others in the same experiment.

² Curvature visible ($10 \pm ^\circ$).

³ Curvature evident ($25 \pm ^\circ$).

⁴ Curvature distinct ($35 \pm ^\circ$).

tissue. The percentage distribution of radioactivity in upper and lower halves of the organ is calculated from the total radioactivity.

The distribution of radioactivity in upper and lower halves of pea roots and shoot pretreated for 1—5.5 hours (experiments 1—4 and 9) was equal although curvature was visible after 90 minutes' stimulation. A symmetric distribution was also found in corn roots pretreated for 2 hours and then stimulated for various lengths of time (experiment 7). The lima bean root

is about 3 to 5 times larger than that of pea and corn, and thus the dividing process and the determination of radioactivity should be more accurate and more sensitive in this plant material. Nevertheless, a similar result was obtained from lima bean roots pretreated for 2 hours and stimulated for different lengths of time (experiments 5 and 6). The percentage distribution of radioactivity in the decapitated corn roots (expt. 8) showed a definite trend of increasing on the lower side in reference to the length of stimulation. However, the fact that prolonged stimulation of plants pretreated the same manner even to 48 hours did not show any bending, but continued to elongate though somewhat less than the control, is difficult to explain.

From these data it is apparent that regardless of length of pretreatment (total absorption), of stimulation (stage of redistribution), and of plant species, an appreciable unequal distribution of radioactivity has not been found in the plants studied.

Several points indicated in Table 1 are also of special interest to plant physiologists: (1) absorption of IAA-1-C¹⁴ increases with time of treatment as shown in the results of experiment 1; (2) the treatment of IAA delays geotropic response from 45 minutes (Ching *et al.* 1956) to approximately 90 minutes or longer in peas and corn, such delay was also found by Rufelt (1954); (3) shoots absorb much less IAA-1-C¹⁴ per unit weight than roots of the same species as indicated by experiments 2 and 9; (4) rate of absorption varies with different plant species.

Discussion

The validity of these experiments depends on the metabolism of exogeneous IAA in plants. The *in vitro* fate of IAA administered into pea tissue has been summarized by Galston (1956) as: (1) a small amount is firmly bound to protein; (2) a much larger portion suffers oxidative degradation to physiologically inactive compound and (3) a small amount remains in free form. Whether similar alteration holds true *in vivo* and at the auxin concentration in present experiments remains for future study. However, for the purpose of speculation, it suffices to consider the results from *in vitro* studies.

The radioactivity extracted from tissue in these experiments probably comes largely from bound IAA-1-C¹⁴ and only a small amount from free IAA-1-C¹⁴ since oxidative degradation of IAA-1-C¹⁴ involves decarboxylation and the decarboxylated product is non-radioactive. Radioactive carbon dioxide produced from the degradation will theoretically evolve from tissue and the small amount possibly fixed during the time limit of these experiments probably is too small to interfere with the determination. Some attempts have

Table 2. *Translocation of radioactivity in IAA-1-C¹⁴ treated seedlings.*

Material and no. of seedlings	Absorption in hr.	Incubation in hr.	Total radio-activity cpm/mg. DW	% distribution			
				root tip (3—5 mm.)	root	hypocotyl (3—6 mm.)	epicotyl
pea (18)	2.5	15	15.8	40.0	33.7	26.3	0
pea (20)	24	—	110.8	45.5	34.0	20.5	
lima bean (16)	22	—	146.3	36.5	45.5	17.1	0.9
corn (80)	22	—	78.3		94.5	3.2	2.3

been made to isolate free IAA from the extract by paper chromatographic technique. At least 6 radioactive spots besides origin have been separated on the chromatogram. Judging by their R_f value, these spots might be the same metabolites found in plants with foliage treatment of IAA-1-C¹⁴ (Fang and Butts 1957). The radioactivity located at IAA spot was found to be only 5—10 % of the total in corn roots pretreated for 22—24 hours, and 0 % for pea roots with the same pretreatment. Roots with shorter duration of pretreatment as applied in the majority of these experiments contain too small amount of radioactivity to work with, thus, it was difficult to determine the validity of these experiments by the amount of free IAA remaining in tissue.

The mobility of the bound IAA-1-C¹⁴ in tissue now becomes the decisive factor although the function of bound auxin is not well understood yet. Some translocation studies revealed that probably the bound IAA-1-C¹⁴ is translocatable (Table 2), but gravity as shown in Table 1 did not alter its distribution.

Reisenner (1957) recently reported that methylene-carbon labeled IAA is not redistributed in geotropically stimulated *Avena* coleoptile tip *in vitro*. Thus, these experiments are amply confirmed and there is probably enough evidence to conclude that lateral redistribution of exogenous IAA by gravitational or geoelectric force in horizontally placed plant organs does not occur.

This finding, however, does not exclude the possibility of gravity induced redistribution of endogenous auxin-complex proposed very recently by Gordon (1957) as the possible lateral transported substances in phototropically induced *Avena* coleoptile. Geotropically induced redistribution of other naturally occurring growth substances (inhibitors and promoters), and/or geoelectrically induced differential production of auxin, if occurring, are not precluded by the result of this study.

Summary

The *in vivo* distribution of administered auxin in geotropically stimulated plant organs was determined by tracing the radioactivity of carboxyl-carbon labelled indoleacetic acid (IAA-1-C¹⁴) in upper and lower halves of horizontally placed root tip or shoot tip. It was found that regardless of the concentration of absorbed auxin, stimulation time and plant species, an appreciable unequal distribution of radioactivity has not been found in the plants studied.

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On the Physiology of Antheridium Formation in the Bracken Fern [*Pteridium aquilinum* (L) Kuhn]

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Introduction

Döpp (1950) showed that an extract from mature prothalli of *Pteridium aquilinum* hastened the formation of antheridia in young prothalli of this same fern species by a few days and in the prothalli of *Athyrium filix mas* by a few weeks. Döpp envisaged the possibility that the extract-induced antheridia were initiated by way of unspecific growth inhibition. This interpretation was prompted by his observation that the extract inhibited growth. It was also compatible with the observations of earlier investigators (see *e.g.*, Prantl 1881) that male prothalli are smaller than female prothalli and that conditions interfering with growth, *e.g.*, low light intensity, crowding and poor mineral supply favor the production of antheridia over that of archegonia. It was possible, however, to obtain highly active preparations that did not inhibit growth (Näf 1956). This and other results led to the conclusion that the extract brings about the formation of antheridia not by way of unspecific growth inhibition but by a specific antheridium-inducing factor.

It was also possible to devise a reliable assay system (Näf 1956) and to define conditions under which an extract of mature prothalli was active to a dilution of 1:31,250 (more than 300 times as active as that obtained by Döpp). Under these same conditions the active factor accumulated to almost as high an activity in the medium. The available information allowed for the calculation that the active factor must be active at a concentration as low as 1.6×10^{-9} .

The factor was shown to be active in inducing antheridia in representatives of 7 subgroups of the family Polypodiaceae and in some, if not all, representatives of 2 further fern families, the Dicksoniaceae and the Schizaeaceae (Näf 1956 and unpublished results). The addition of the active factor hastened the onset of antheridium formation in most of these fern species by a minimum of 4 weeks. It also led to the formation of antheridia in a number of fern species that failed to form them spontaneously under the prevailing conditions of culture.

The present investigation attempts to define more closely the relationship between antheridium formation and the antheridial factor in the gametophyte of *Pteridium aquilinum*. It also deals with new observations on antheridium formation and their interpretation in terms of the antheridial factor.

Methods

Method of culture

The medium described by Moore (1903) was used throughout the investigation. This medium, which is composed of NH_4NO_3 , 0.5 g.; KH_2PO_4 , 0.2 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g.; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g.; ferric tartrate, 5 mg.; H_2O , 1 l., was supplemented with Hoagland's A—Z solution of microelements. In most of the studies the gametophytes were grown in 125-ml. flasks containing 33 ml. of medium which, unless specified otherwise, was solidified by one per cent agar. In some instances 50-ml. flasks were used instead, to which 10 ml. of agar-solidified medium were added. The media, including those containing antheridial factor, were sterilized by autoclaving for 15 minutes at 20 lbs. atmospheric pressure. The prothalli were cultivated at a temperature of $23 \pm 0.5^\circ\text{C}$ under continuous illumination by "standard cool white" G. E. fluorescent lamps at an intensity of ca. 200 f.c.

Sterilization and inoculation of the spores

About 3 mm.³ of spore material were put into a 5-ml. glass tube and shaken with a wetting agent (2 drops of 25 per cent aerosol OT per 100 ml. of water) until the spores were in suspension. The suspension was filtered through hard filter paper, the wet spore mass scraped off with a small spatula and transferred to the bottom of a sterile 5-ml. glass tube. Two drops of hypochlorite solution (0.5 per cent by weight) were then added to the spores (the commercial product Clorox, at a dilution of 1 : 10, was used). Effective sterilization was stopped after 15 to 25 seconds by the addition of ca 4 ml. of sterile water, the length of sterilization depending on the amount and type of contamination found in the spore sample. The resulting suspension was transferred to a 20-ml. test tube and diluted to a final volume of 10 ml. By means of small sterile pipettes, 2 to 6 drops of this suspension were added to the culturing

flasks, the number of drops depending on the size of the culturing flasks and the desired concentration of the inoculum. The suspension was distributed evenly over the agar surface by gently tilting the flask in all directions. (Wetting of the spores by aerosol water prior to sterilization shortened the time necessary for effective sterilization.)

Assay

The antheridial factor was assayed against gametophytes of *Onoclea sensibilis*. Gametophytes of this fern species proved suitable because they did not form antheridia at any stage of development under the conditions of culture described but responded readily and sensitively with the formation of antheridia when antheridial factor was added to the medium.

Antheridium-inducing activity was assayed by means of a dilution series.

If an experiment required the addition of antheridial factor, the liquid medium obtained from 7-week-old cultures of *Pteridium aquilinum* (mostly active to a dilution of about 1 : 30,000) was used.

Experimental Results

1. Course of antheridial factor secretion and of other events in the development of *Pteridium* gametophytes

The activity of extracts from mature *Pteridium* prothalli and that of the supporting medium was determined earlier (Näf 1956). It remains to study the course of antheridial-factor secretion from its onset. This problem was approached by determining the rate at which the antheridial factor accumulates in the medium. For this purpose the activity of the *Pteridium* medium was assayed at frequent intervals against prothalli of *Onoclea sensibilis* by means of the dilution series: 1/2, 1/4, 1/8, 1/16, etc. Each determination was based on a sample that combined the media of four randomly chosen cultures (the prothalli were carefully scraped off the agar surface, and the media combined after they had been dissolved on a hot water bath). The activities of the media were entered into Figure 1 as the dilutions which induced antheridia in 50 per cent of the gametophytes, or the dilutions which most closely approximated this 50 per cent response (these dilutions were either the terminal ones or the ones preceding them). The values are plotted as the logarithms (base 2) of the dilutions at which these 50 per cent responses were obtained.

Separate observations served to relate the course of antheridial-factor secretion to the first appearance of heart-shaped, antheridium-bearing and archegonium-bearing prothalli in the culture. The criterion used for the appearance of antheridium initials was the emergence of semicircular out-

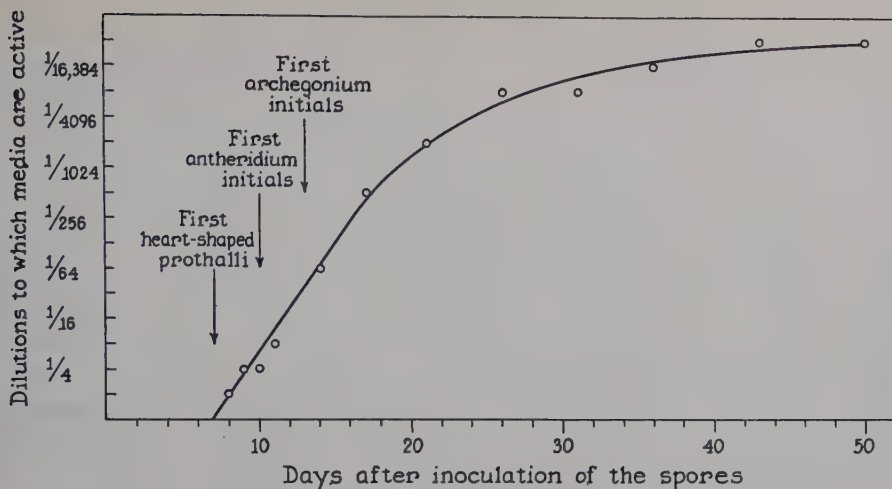


Figure 1.

growths which were not as yet cut off from the vegetative mother cells. They could be distinguished from rhizoid initials by their position (the area of antheridium formation extends farther forward) and by a clearing zone around the front of the outgrowth (resulting from a slight retraction of the chloroplasts from the cell wall). An archeogonium was judged to be initiated at the four-cell stage and the prothalli were considered heart-shaped with the first appearance of a notch in the center of the growing region. The dates recorded for these events in Figure 1 are based on daily observations.

A second experiment was undertaken to relate the course of antheridial-factor secretion to these and other events of development in more detail. This was accomplished by determining the percentages of germinated, heart-shaped, antheridium-bearing and archeogonium-bearing prothalli over a period of time, at frequent intervals. The experiment was terminated when the cultures were 20 days old because the prothalli were then so entangled that reliable counts could no longer be obtained.

The percentage values in Figures 1 and 2 are based on observations on 100 randomly picked prothalli in each of four randomly picked flasks; the final values represent the averages of the percentages calculated for each of the four flasks. New cultures were used at each interval.

Figures 1 and 2 illustrate some basic features of gametophyte development necessary to understand the sequence of developmental events associated with the secretion of the antheridial factor. The presence of antheridial factor in the medium is first detected 2 days before antheridium initials can be

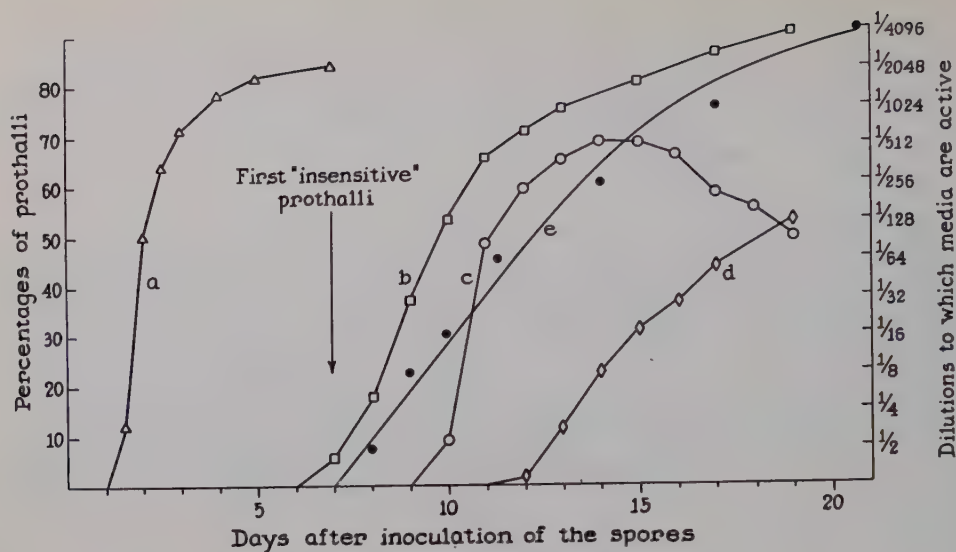


Figure 2.

- a. Percentages of germinated spores.
- b. Percentages of heart-shaped prothalli.
- c. Percentages of prothalli that bear antheridia (or their initials).
- d. Percentages of prothalli that bear archegonia (or their initials).
- e. Dilutions of *Pteridium* medium which brought about the formation of antheridia in 50 per cent of the assay prothalli.

For explanation of arrow with annotation "first insensitive prothalli", see section three of Experimental Results.

discerned; the close sequence of the two events is again indicative of a causal relationship between them. The two Figures also show that the first detection of antheridial factor in the medium follows the first appearance of heart-shaped prothalli by 1 day and precedes the first appearance of archegonium-bearing prothalli by 4 days (Figure 2) or 5 days (Figure 1).

Once started, the accumulation of antheridial factor preceeds at a progressively faster rate until about 8 days after its first detection. At that time the medium is active to a dilution of about 1:512. The accumulation of antheridial factor continues, but at a gradually slower rate. The maximum activity of the medium is reached about 7 weeks after inoculation of the spores; the medium then can in most cases be diluted 32,768 times, occasionally even 65,536, and still retain activity.

The steep slope of the curve representing antheridium-bearing prothalli (Figure 2), as compared to the lesser slopes of the curves representing heart-shaped and archegonium-bearing prothalli, is of interest. It may be under-

stood on the assumption that the most rapidly developing individuals of the gametophyte population begin to secrete the antheridial factor first, thus causing the formation of antheridia in the laggard prothalli which have not themselves proceeded to produce the factor.

2. *Three types of prothalli in cultures of Pteridium aquilinum*

Mature gametophyte cultures of *Pteridium aquilinum*, and of many other homosporous fern species, contain two types of prothalli. A minority of them bear antheridia only and are for this reason designated the male prothalli (tiny structures of irregular outline owing to their lack of a meristem). The majority, though, are made up of the so-called female prothalli (with well defined heart shape and of much larger size) which bear archegonia only (Campell 1918) or rarely, mostly under atypical conditions of culture, also a few antheridia (Mottier 1910, Wuist 1910). These observations on mature cultures led to the concept that the so-called female prothalli formed archegonia only under normal conditions of culture, or but rarely also a few antheridia (Campell 1918, Mottier 1910, Wuist 1910, Döpp 1937, 1950). In contrast, Czaja (1924) came to the conclusion that the female gametophytes form antheridia regularly, but discontinue their formation when they attain the archegonial stage.

Figure 2 shows that the proportion of antheridium-bearing prothalli reaches a maximum of about 68 per cent 14 to 15 days after inoculation of the spores and then begins to decline. Since the percentage of male prothalli in mature cultures never surpassed 5 per cent under the prevailing conditions of culture, the conclusion is drawn that the archegonial phase of many "female" prothalli is actually preceded by an antheridial phase.

While a majority of the archegonium-forming prothalli have a prior phase of antheridium formation, others are without it. Observations at short intervals of time disclosed that the biggest, *i.e.*, the most rapidly growing and developing individuals of the gametophyte population, were without antheridia from the first appearance of these sex organs (in more slowly growing prothalli) to the formation of archegonia by the most quickly developing gametophytes. It is concluded that the most rapidly developing individuals of the gametophyte population (about 20 per cent under the conditions of culture) proceed to form archegonia without the prior formation of antheridia. An interpretation of this phenomenon in terms of the antheridial factor is deferred to the discussion.

The question arises, When do the antheridia formed on archegonium-forming prothalli with a prior phase of antheridium formation disintegrate? The following observations are of pertinence in this connection: The gametophytes which attained the archegonial phase first (12—14 days after

inoculation) invariably lacked antheridia (prothalli without a prior antheridial phase). On the other hand, most of the prothalli that initiated the first archegonium between 14 and 15 days after inoculation bore antheridia. In 16-day-old cultures they could also be observed on many prothalli that had just initiated the second archegonium, and in 17-day-old cultures even on some which had just initiated the third archegonium (the prothalli initiated new archegonia at the rate of about one per day at this age of the culture). Corresponding observations were made in still older cultures: Most of the prothalli which had just initiated the first archegonium, many of those which had just initiated the second, and some which had just initiated the third archegonium, bore antheridia. Prothalli bearing four or more archegonia, though, were invariably without antheridia (as judged from observations ranging from 15 days after inoculation when prothalli with four archegonium initials first occurred, to 21 days after inoculation when the maximum number of archegonia per prothallus reached ten). The conclusion is drawn that the last of the antheridia produced on archegonium-forming prothalli with a prior phase of antheridium formation disintegrate when the prothalli have initiated from one to three archegonia. The persistence of antheridia in prothalli bearing up to three archegonium initials accounts, at least in part, for the observation that the decline in the proportion of antheridium-bearing prothalli does not set in until about 3 days after the first appearance of archegonium-bearing prothalli (Figure 2). Another part of the lag must be traced to the absence of a prior antheridial phase in the prothalli which attain the archegonial phase first.

As shown above, the antheridia formed at an early stage of development disintegrate shortly after the prothalli attain the archegonial phase. Some of the archegonium-forming prothalli, though, may resume antheridium formation secondarily at late stages of development after they have initiated numerous archegonia. In accordance with the observations of Mottier (1910), such "secondary" antheridia are never found on the main bodies of the gametophytes. Instead they arise on some of the basal outgrowths which a number of the prothalli form at late stages of development. The simultaneous occurrence of the two sex organs on gametophytes of *Pteridium aquilinum* is thus encountered on young prothalli bearing up to three archegonium initials, and again on the basal outgrowths of very old prothalli.

In summary, a culture of *Pteridium aquilinum* contains three types of prothalli:

Archegonium-forming prothalli without a prior antheridial phase (the most rapidly growing and developing individuals of the gametophyte population).

Archegonium-forming prothalli with a prior antheridial phase (the bulk of the remaining prothalli).

A few of the so-called male prothalli which produce antheridia only throughout the life of the culture (preliminary investigations indicate that they arise from the most slowly growing individuals of the gametophyte population).

3. Loss of sensitivity to the antheridial factor.

a) In *Onoclea sensibilis*

As shown above, the antheridia formed at an early stage of development disintegrate as the prothalli attain the archegonial phase. An attempt to explain this phenomenon in terms of the antheridial factor led to the hypothesis that the maturing prothalli become insensitive to it, *i.e.*, that they lose the ability to respond with antheridium formation to the presence of the antheridial factor. This hypothesis was put to a test first on the prothalli of *Onoclea sensibilis* which are particularly suited to such an investigation because they fail to form antheridia spontaneously under the prevailing conditions of culture but form them readily in response to added antheridial factor (Näf 1956). Prothalli of this fern were transferred at regular intervals from cultures grown in 125-ml. flasks to new medium containing the antheridial factor at 1/2; 1/10, 1/50, *etc.*, to 1/31,250 full strength Pteridium medium that was active to a dilution of about 1 : 30,000. Transfer occurred at intervals of 2 days starting 8 days after inoculation of the spores. Twenty prothalli were transferred at each time interval and each concentration of antheridial factor (5 prothalli to each of four 50-ml. flasks). Results were read 5 days after transfer since preliminary experiments had shown this to be a suitable period for testing whether or not the prothalli were capable of

Table 1. Number of prothalli (out of 20) that formed antheridia when transferred from basic medium to medium containing antheridial factor at different intervals following inoculation of the spores.

Dilution of Pteridium medium	Interval				
	8 days	10 days	12 days	14 days	16 days
1/2	20	20	7	2	0
1/10	20	19	6	0	0
1/50	20	20	5	1	0
1/250	20	20	6	2	0
1/1,250	20	20	5	2	0
1/6,250	20	19	5	1	0
1/31,250	7	6	1	0	0
Control	0	0	0	0	0

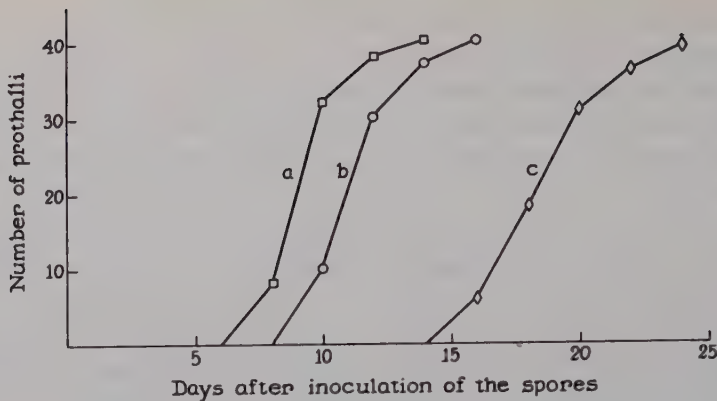


Figure 3.

- a. Percentages of prothalli that have attained heart-shape.
- b. Percentages of prothalli that have become insensitive to the antheridial factor.
- c. Percentages of prothalli that have initiated one or more archegonia.

responding with antheridium formation. Table 1 records the results as number of prothalli (out of 20) which at the indicated age, and activity of the antheridial factor, responded with antheridium formation.

Table 1 shows that, whereas nearly all 10-day-old prothalli were sensitive to the antheridial factor, nearly all 14-day-old gametophytes were insensitive to it. It may be added that the prothalli obtained from an earlier spore sample did not start to become insensitive until 15 days after inoculation, probably because they grew and developed more slowly (Näf 1956). It may thus be concluded that a population of *Onoclea* gametophytes becomes insensitive to the antheridial factor within a period of about 4 days. Care was taken in the above experiment to transfer random samples of the gametophyte population which thus included prothalli of varying sizes and stages of development. When the experiment was repeated with the transferred prothalli as nearly as possible of equal and of medium size, then this loss of sensitivity occurred within 2 days for 9 out of 10 gametophytes. This period of 2 days is likely to approximate the interval within which the individual gametophyte becomes insensitive to the antheridial factor; following this interval a gametophyte fails to form antheridia even in response to the highest available concentration of antheridial factor, *i.e.*, a concentration 15,000 times higher than that sufficient to induce antheridia in prothalli just 2 days younger.

The last experiment of this section relates the onset of this loss of sensitivity to the time sequence of two other major events in the development of *Onoclea* gametophytes: attainment of heart-shape and onset of archegonium formation (Figure 3). Loss of sensitivity (curve b) was obtained as above except that

the test involved only one concentration of antheridial factor (1/50 full strength of 7-week-old *Pteridium* medium that was active to a dilution of about 1:30,000). Curve a records, at intervals of 2 days, the numbers of prothalli that had attained heart-shape, while curve c shows the numbers of prothalli that had initiated archegonia (out of 40 observed at each interval).

The results recorded in Figure 3 show that the prothalli become insensitive to the antheridial factor about 2 days after they attain heart-shape and *ca.* 6 days before they begin to form archegonia.

b) In *Pteridium aquilinum*

It remained to be determined whether maturing prothalli of *Pteridium aquilinum* also lose their sensitivity to the antheridial factor. This information was not so easily obtained with this fern species as for *Onoclea sensibilis*, because cultures of *Pteridium aquilinum* formed antheridia spontaneously. The procedure used above could, however, be modified in such a way that the onset of this loss of sensitivity could be timed at least among the most rapidly developing individuals of the gametophyte population which attain the archegonial phase without first forming antheridia. The pertinent information was obtained as part of the experiment recorded in Figure 2.

Antheridial factor (1 ml. of 1/50 dilution of the liquid medium of 7-week-old *Pteridium* cultures that was active to a dilution of about 1:30,000) was added to young cultures of *Pteridium aquilinum* at intervals of 1 day (five 125-ml. flasks per interval) starting with 5-day-old cultures, while the controls received an equal amount of fresh medium. The results were read 5 days after the addition of antheridial factor.

Inspection of the cultures treated with the factor 5 or 6 days after inoculation of the spores disclosed that all prothalli had responded with antheridium formation. In the cultures supplied with antheridial factor 7 days after inoculation, though, a few of the developmentally most advanced prothalli (about 2 or 3 out of 100) failed to form antheridia. In the cultures treated with antheridial factor 8 days, 9 days, or 10 days after inoculation, the percentage of such unresponsive prothalli increased to about 5, 9, and 17 per cent, respectively. Thus, in 10-day-old cultures the percentage of prothalli that had become insensitive about equaled the percentage of prothalli (*ca.* 20 per cent) which proceeded to form archegonia without a prior phase of antheridium formation (see previous section). Since the remaining prothalli all form antheridia spontaneously, the effect of the added antheridial factor cannot be followed beyond this stage.

It is therefore concluded that the most rapidly developing individuals of the gametophyte population become insensitive to the antheridial factor

7 days after inoculation (see arrow in Figure 2 with connotation: "first insensitive prothalli").

It can be further observed in Figure 2 that the first heart-shaped prothalli (about 8 per cent) are detected on the same day that the first "insensitive" prothalli (about 2—3 per cent) can be discerned. The observation that the percentage of heart-shaped prothalli surpasses that of insensitive prothalli suggests that the gametophytes of *Pteridium aquilinum* lose their sensitivity to the antheridial factor shortly after they attain heart-shape. The following experiment yielded more definitive information regarding the time sequence of these two events: 100 randomly chosen prothalli (25 out of each of four 9-day-old cultures) were transferred, one per (50-ml.) flask, to antheridial-factor-containing medium (1/50 full strength medium of 7-week-old *Pteridium* cultures that was active to a dilution of about 1 : 30,000). Prothalli isolated at such an early stage of development all failed to form antheridia spontaneously but formed them readily in response to added antheridial factor (for detailed account of this phenomenon, turn to last section of Experimental Results). This isolation technique thus enables the investigator to more precisely time the loss of sensitivity in relation to the attainment of heart-shape (each flask was marked to show whether or not the prothallus was heart-shaped when transferred to the antheridial-factor-containing medium; total of heart-shaped prothalli: 39 out of 100). Examination of the prothalli for antheridium formation occurred 5 days following exposure to the antheridial factor.

The prothalli that failed to attain heart-shape at the time of transfer all formed antheridia. Of the prothalli which had achieved heart-shape, though, only 15 responded with antheridium formation (microscopic examination indicated them to be those that had assumed heart-shape most recently). It is therefore concluded that the gametophytes of *Pteridium aquilinum*, like those of *Onoclea sensibilis*, do not lose their sensitivity to the antheridial factor until after they attain heart-shape.

It is further noted (Figure 2) that the presence of antheridial factor in the medium is not detected until 1 day after the prothalli have become insensitive to it. This suggests, but does not establish, that the prothalli become insensitive to the antheridial factor before they begin to elaborate it. This question will be dealt with again in the discussion when further information can be brought to bear.

4. Duration of antheridial factor secretion

Figure 2 shows that the activity of the medium continues to increase at a fast rate as more and more prothalli stop forming antheridia and begin to produce archegonia instead. This suggests that the gametophytes continue

Table 2. Number of media (out of 20 at each interval) that are active to the indicated maximal dilutions.

Dilution of medium	Time		
	3 days	13 days	23 days
1/2	17	—	—
1/10	3	2	—
1/50	—	16	2
1/250	—	2	12
1/1,250	—	—	6

to elaborate the antheridial factor as they attain the archegonial phase. It must be pointed out, however, that even mature cultures contain a small percentage of gametophytes (the so-called male prothalli) which form antheridia throughout the life of the culture. Accordingly, it may be these male prothalli rather than the archegonium-bearing prothalli which are responsible for the continued elaboration of antheridial factor by maturing cultures of *Pteridium aquilinum*. A decision between these two possibilities must be based on experimentation with isolated gametophytes.

Sixty randomly picked gametophytes (15 out of each of 4 flasks) were transferred from 9-day-old cultures to new medium, one per 50-ml flask, each of them containing 10 ml. of nutrient. Seven days afterward they were transferred a second time, again one per 50-ml. flask. Microscopic examination which preceded the second transfer of each prothallus showed that 58 out of the 60 had initiated from 1 to 4 archegonia; the remaining 2 gametophytes were of advanced heart-shape but had not as yet initiated the first archegonium. The antheridial factor activities of the media supporting these isolated gametophytes were determined 3, 13, and 23 days after the second transfer (for 20 prothalli at each interval).

Table 2 shows that each of the 20 media assayed 23 days after the transfer was at least 5 times more active than any of the 20 media assayed 3 days after transfer. It was therefore concluded that the prothalli actually continue to produce the antheridial factor while they form archegonia, *i.e.*, after they have discontinued the formation of antheridia and after they have become insensitive to the antheridial factor.

5. The effect of inoculation density on antheridium formation

Much of the antheridial factor produced by the prothalli was shown to be secreted into the medium. The secreted factor may be taken up again and bring about the formation of antheridia in prothalli which themselves have not as yet started to produce it. This accounts for the steep slope of the curve

representing antheridium-bearing prothalli in Figure 2 as compared to the lesser slopes of the curves representing heart-shaped and archegonium-bearing prothalli, respectively. Conditions that influence the amount of secreted antheridial factor, inoculation density *e.g.*, may thus be expected to affect antheridium formation. Accordingly, the effect of inoculation density on antheridium formation, specifically on the onset of antheridium formation and on the percentage of prothalli which proceed to form archegonia without a prior phase of antheridium formation, was studied.

A dense, sterile, spore suspension was obtained and sterily diluted to give the dilution series 1/1, 1/5, 1/25, 1/125, 1/625. Eight drops of suspension were then added to each of the 50-ml. flasks at each strength of spore suspension.

The numbers of prothalli at the lowest density of inoculation (1/625) were counted 15 days after inoculation in each of 15 flasks and the average determined as 2.8. The numbers of prothalli at higher densities of inoculation were calculated by multiplying this value with the respective dilution factors.

"Onset of antheridium formation" is entered into Table 3 as the number of days that elapsed between spore inoculation and the first detection of antheridium initials.

The percentages of archegonium-forming prothalli without a prior phase of antheridium formation were obtained by determining the minimal percentages of antheridium-free prothalli found during the development of the culture. For this purpose the percentages of antheridium-free prothalli were obtained 3, 4, 5, and 6 days after antheridia could first be seen at the various densities of inoculation (preliminary investigations showed the minimum to occur within this period of time). The minimal values among these four percentages were then selected and, for each density of inoculation, entered in column 2 (Table 3).

Careful observations showed that the prothalli that initiate antheridia first do not become free of them before the more slowly developing prothalli have also formed antheridia. Accordingly, the maximal number of antheridium-bearing prothalli corresponds very closely to the total number of prothalli that form antheridia before they proceed to initiate archegonia. Conversely, the minimal percentages of antheridium free prothalli correspond very closely to the proportion of prothalli which proceed to produce archegonia without the prior formation of antheridia.

Preliminary observations indicated that density of inoculation, besides affecting antheridium formation, also influenced the rate of vegetative growth. Counts of the numbers of cells present in 8-day-old gametophytes were therefore made at each density of inoculation (rhizoidal cells were not

Table 3. *Relation between density of cultures and antheridium formation.*

Number of prothalli per flask (10 ml. of medium)	Onset of antheridium formation (in days after inoculation of spores)	Proportion of prothalli that proceed to form archegonia without first forming antheridia	Number of cells in 8-day-old gametophytes
8750.0	9	18.1 % \pm 0.9	34.8 \pm 0.7
1750.0	10	20.2 % \pm 0.9	32.0 \pm 0.5
350.0	12	31.7 % \pm 1.0	27.8 \pm 0.8
70.0	13	43.8 % \pm 1.7	23.1 \pm 0.9
14.0	15	57.7 % \pm 1.1	18.8 \pm 0.4
2.8	—	—	—

counted; the 8-day-old prothalli did not at that early stage bear any antheridial cells at any density of inoculation).

Each value recorded in Table 3 is based on observations, or cell counts, of 100 randomly picked prothalli in each of four randomly picked cultures. The standard error is representative of the spread between the values obtained for each of the four flasks.

Table 3 shows that increasing the density of inoculation greatly increased the average number of cells contained by 8-day-old gametophytes. Further investigations demonstrated that increasing the density of inoculation does not hasten the rate of spore germination. It may thus be concluded that increased density of inoculation increases the rate of early vegetative growth. This probably means that the prothalli, like many microorganisms, must condition the medium before they attain an optimal rate of growth.

The effects on antheridium formation are equally pronounced: a decline in the density of inoculation correlates with a delay in the onset of antheridium formation and an increase in the proportion of prothalli that attain the archegonial phase without the prior formation of antheridia. This raises the possibility that all prothalli will lack the antheridial phase if they are grown one per flask.

The hypothesis was put to a test by transferring 240 randomly picked prothalli from 9-day-old cultures to new medium, one per 50-ml. flask, each of them containing 10 ml. of medium. The isolation of these prothalli occurred 2 days prior to the detection of antheridial factor in the medium and 4 days before the first antheridium initials could be discerned in the culture. Examination for antheridium formation occurred at intervals of 2 days, starting 2 days following their isolation. Observations were continued until all prothalli had initiated archegonia.

All prothalli remained free of antheridia regardless of the interval at which they were examined. It may be added that antheridia did appear on some of

the prothalli after they had initiated numerous archegonia. Such "secondary" antheridia, though, were found not on the main bodies of the gametophytes but on the basal outgrowths which some of them formed at late stages of development (see also section 2 of Experimental Results).

The experiment was repeated by isolating the prothalli on a minimal amount of medium (0.3 ml. contained in small test tubes) rather than the 10 ml. used above; the result was unchanged.

The hypothesis is thus confirmed that all gametophytes proceed to form archegonia without the prior formation of antheridia if they are grown one per flask.

Discussion

The reported studies show that all but the most rapidly developing individuals among the archegonium-forming prothalli of *Pteridium aquilinum* have an early phase of antheridium formation which, however, is terminated as the prothalli attain the archegonial phase.

Why do the prothalli discontinue the formation of antheridia? It is clear that this question must be asked in relation to the antheridial factor which controls the induction of antheridia in many species of ferns. Figure 2 shows that the antheridial factor becomes available at progressively higher concentrations as more and more prothalli stop forming antheridia and begin to produce archegonia instead. Indeed, studies on isolated prothalli showed that they continue to produce large amounts of the factor long after they attained the archegonial phase. Discontinuation of antheridium formation could not therefore be ascribed to a discontinuation of antheridial factor production. The alternative hypothesis may be suggested, that the maturing prothalli become insensitive to the antheridial factor, *i.e.*, that they lose the ability to respond with antheridium formation to the presence of that factor.

The studies reported here actually showed that the prothalli of *Pteridium aquilinum* as well as those of *Onoclea sensibilis* lost their sensitivity to the antheridial factor shortly after they attained heart-shape. In the case of *Onoclea* prothalli, this loss of sensitivity was found to occur within a period of about 2 days; following this period they failed to form antheridia even in response to the highest available concentration of antheridial factor, *i.e.*, a concentration 15,000 times higher than that sufficient to induce antheridia in prothalli just 2 days younger. Investigations on the physiological basis of the phenomenon described here are in progress.

Further insight into the relationship of the antheridial factor to antheridium formation was derived from the observation that the most rapidly developing

individuals of the gametophyte population failed to form antheridia prior to the initiation of archegonia. An attempt to explain this phenomenon led to the hypothesis that the prothalli became insensitive to the antheridial factor before they started to produce it at effective concentrations. On this assumption, the antheridia a gametophyte forms would arise in response to antheridial factor that is secreted into the medium by more rapidly developing individuals of the gametophyte population (which have themselves already become insensitive to it). The individuals of the gametophyte population that develop most rapidly would, according to this concept, fail to form antheridia because they alone are without a supply of antheridial factor while they are still sensitive to it.

If this hypothesis be correct, then:

a) All prothalli should form antheridia prior to the formation of archegonia if all are supplied with antheridial factor while they are still sensitive to it. Application of antheridial factor to the medium 5 days after inoculation of the spores actually led to this result.

b) In contrast, all prothalli should be without antheridia if grown one per flask. This was shown to be the case even when the prothalli were grown on a minimal amount of medium.

These results are considered to confirm the thesis that the prothalli do not begin to elaborate the antheridial factor at effective concentrations until after they have become insensitive to it.

It would be of interest to know what advantage, if any, accrues to the plant from this delayed onset of antheridial-factor production. The following considerations may provide a clue: If the prothalli were to form effective amounts of the antheridial factor before they became insensitive to it, then all gametophytes would first form antheridia, including the most rapidly developing ones which otherwise proceed to form archegonia without the prior formation of antheridia. Such antheridium formation retards the attainment of the archegonial phase, first, by diverting "growth potential" from the formation of vegetative cells to that of antheridial cells, second, because it tends to interfere with the development of the meristem that is characteristic of the archegonium-bearing prothallus (The mechanism of this interference will be detailed in a subsequent report). Accordingly, the formation of archegonia by the most rapidly developing prothalli, and thereby the first appearance of archegonia in the culture, would be postponed. The failure of the prothalli to produce effective amounts of the antheridial factor before they become insensitive to it may thus be considered a device to keep the time lag between the appearance in the culture of antheridia and of archegonia (see Figures 1 and 2) to a minimum, thereby hastening the onset of sexual reproduction.

The preceding discussion offers an explanation for the observed effects of inoculation density on antheridium formation (Table 3). Increasing the density of inoculation increases the number of prothalli that secrete the antheridial factor, thereby hastening the attainment of the threshold concentration necessary for antheridium formation. The greater proportion of antheridium-forming prothalli at higher density of inoculation may be considered the result of this (fewer prothalli become insensitive to the antheridial factor before it becomes available at sufficient concentration). The earlier onset of antheridium formation at higher densities of inoculation might again be referred to the greater number of secreting prothalli. On the other hand, increased densities of inoculation also greatly increased the rate of early vegetative growth and, most likely, hastened the attainment of the developmental stage at which the prothalli begin to secrete the antheridial factor. It thus appears that the earlier onset of antheridium formation at increased densities of inoculation is referable as much to an earlier onset of antheridial-factor secretion (faster rate of prothallial growth) as to the greater amount of antheridial factor produced (greater number of secreting prothalli).

The demonstration that high density of inoculation promotes growth takes issue with the many statements in the literature that conditions interfering with growth, *e.g.*, crowding, promote antheridium formation. The implication of such statements that high density of inoculation (crowding) interferes with vegetative growth no doubt applies to older cultures because of competition for nutrients and the possible secretion of substances harmful to growth. As regards the early growth of *Pteridium* prothalli, the relationship between crowding and rate of growth is clearly the reverse. Since the measurements of Table 5 were obtained just one day before antheridium initials at the highest density of inoculation could first be seen, the data indicate that here promotion (earlier onset) of antheridium formation is linked to conditions that favor rather than interfere with vegetative growth.

Summary

The relation of the antheridial factor to antheridium formation in *Pteridium aquilinum* was further investigated. Its elaboration by the prothalli appears to set in shortly after they attain heart-shape. Its presence in the medium is first detected 2 days prior to the appearance of antheridium initials.

As observed by earlier investigators, the prothalli discontinue the formation of antheridia as they proceed to form archegonia. The prothalli continue, however, to produce large amounts of the antheridium-inducing factor. Discontinuation of antheridium formation cannot therefore be ascribed to a

discontinuation of antheridial-factor production; instead it results from a loss of sensitivity to it. The prothalli of *Onoclea sensibilis* become insensitive to the antheridial factor within a period of about 2 days. After the gametophytes have become insensitive, they fail to form antheridia even when supplied with antheridial factor at a concentration 15,000 times higher than the concentration necessary to induce antheridia in prothalli just 2 days younger.

Observations showed that the archegonium-forming prothalli of *Pteridium aquilinum* must be subdivided into two types: (1) those with, and (2) those without a prior phase of antheridium formation. The prothalli of the second type arise from the most rapidly growing and developing individuals of the gametophyte population.

The prothalli of *Pteridium aquilinum* start to become insensitive to the antheridial factor before they begin to produce it at effective concentrations. Thus the antheridia which a prothallus forms before it attains the archegonial phase do not arise in response to antheridial factor produced by itself. Instead they arise in response to antheridial factor secreted into the medium by more rapidly developing individuals of the gametophyte population which themselves have already become insensitive to it.

A decrease in the density of inoculation delays the onset of antheridium formation and raises the percentage of prothalli that proceed to form archegonia without the prior formation of antheridia. If grown singly, all individuals of the gametophyte population fail to form antheridia prior to the formation of archegonia. Lowering the density of inoculation also slows the rate of early vegetative growth.

All prothalli are sensitive to the antheridial factor at an early stage of development. All prothalli that attain heart-shape will produce the antheridial factor and lose their sensitivity to it.

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Action du glutathion sur la morphologie et l'activité auxines-oxydasique de tissus cultivés *in vitro*

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Avant-propos

Le glutathion a été considéré généralement comme un inhibiteur de la croissance des tissus végétaux (1, 10). Toutefois, il provoque une accélération du développement des cals de tissus tumoraux cultivés *in vitro* (9) et c'est en connaissant les modifications qu'il subit *in vivo* (4) qu'on a été amené à envisager l'étude de son action parallèlement à celle des hormones de croissance (5). On a constaté, à ce propos, qu'une forte application d'auxines accroît, dans les tissus traités, la teneur en GSH et que la forme GSSG entraîne une nette inhibition de croissance.

But du travail

Nous avons cherché à mettre en évidence l'action du GSH/GSSG sur le métabolisme auxinique, en précisant le rôle de ce corps sur la destruction *in vitro* et *in vivo* des auxines. Ces essais ont porté sur des fragments de carotte cultivés *in vitro* dont les besoins nutritifs sont connus (2, 3) et où l'activité des enzymes de croissance a déjà été étudiée (6).

Observations

On peut noter, après quelques jours de culture déjà, que les néoformations qui apparaissent sur les fragments n'ont pas la même forme si le milieu contient du glutathion (1.10^{-7} à 1.10^{-4} M) ou s'il en est privé (Figure 1). Et malgré

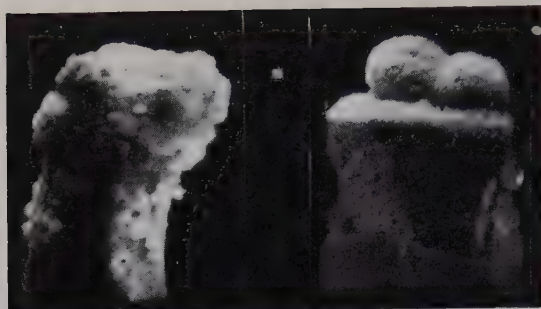


Figure 1 Aspect morphologique de fragments de carotte cultivé *in vitro* (Cultures primaires de 30 jours) dans le milieu de Heller.

A gauche: témoin. A droite: milieu + glutathion à 1.10^{-6} M.

l'extrême variabilité morphologique des massifs cellulaires qui apparaissent sur les fragments sultivés *in vitro*, il nous est possible de déceler quelques modifications de structure très caractéristiques. Dans le cas où les tissus sont cultivés sur milieu enrichi en glutathion, on peut remarquer que les proliférations "s'organisent" en massifs sphériques typiques., alors que les témoins ont des cellules qui s'étalent irrégulièrement à la surface des fragments. En outre la croissance de ces jeunes tissus paraît nettement activée (mais des essais sur des cultures secondaires, où les mesures sont plus faciles, sont actuellement en cours qui permettront de préciser l'action exacte du glutathion sur la croissance des cultures *in vitro*).

L'analyse de la destruction *in vitro* (activité auxines-oxydasique) des extraits de tissus a été entreprise selon notre technique habituelle (7), basée sur l'emploi du réactif de Salkowski-Tang and Bonner modifié.

Nous avons entrepris deux séries de déterminations:

1) Des extraits enzymatiques sont préparés à partir de néoformations développées sur des tissus cultivés en présence ou en absence de glutathion. Il s'agit ici de cultures primaires et le but est d'étudier l'action jouée par le glutathion, incorporé au milieu de culture, sur la destruction des auxines. Cette série d'essais nous permettra de mettre en évidence l'action *in vivo* du glutathion sur l'activité auxines-oxydasique.

2) Des extraits enzymatiques sont préparés comme précédemment, mais à partir de néoformations développées sur des tissus cultivés sur milieu habituel sans trace de glutathion. Le glutathion est ajouté à l'extrait lui-même et son action est étudiée immédiatement. Cette série d'essais nous entraînera à étudier l'action *in vitro* du glutathion sur l'activité auxines-oxydasique.

Essais 1. Effet *in vivo* du glutathion sur la destruction auxinique

A 2 ml d'extrait sont mélangés 6 ml de solution tampon $\text{KH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$ (pH 6,1) et au temps 0 on ajoute 2 ml d'une solution d'acide β -indolyl-acétique

Tableau 1. Action *in vivo* du GSH/GSSG sur l'activité auxines-oxydasique.

Concentrations en GSH/GSSG du milieu de culture <i>M</i>	μg d'ABIA détruits rapportés pour 0,1 mg de protéines N et par 60 mn	% d'inhibition d'activité auxines-oxydasique
1.10 $^{-\infty}$	6,43	0,00
1.10 $^{-7}$	6,40	0,47
1.10 $^{-6}$	6,02	6,22
1.10 $^{-5}$	5,12	20,39
1.10 $^{-4}$	5,00	22,24

Chaque chiffre est une moyenne de 80 mesures (10 extraits différents de 20 ml chacun et 8 analyses par extrait).

(ABIA) à 1.10 $^{-3}$ M. Après une incubation de 60 mn, on prélève 2 ml du mélange qu'on ajoute à 8 ml du réactif (FeCl_3 1,5 n (3 ml); H_2SO_4 1,84/97 % (60 ml); H_2O bidistillée et déionisée (100 ml). La titration se fait à 22,5°C \pm 0,2 par technique photocolorimétrique (5350 Å \pm 150).

Les résultats reportés en fonction de la concentration du milieu en glutathion sont exposés dans le Tableau 1. Ils indiquent que le glutathion est capable de bloquer partiellement l'activité auxines-oxydasique et par conséquent de favoriser ainsi l'accumulation des auxines dans les néoformations de tissus cultivés *in vitro*. Cette action *in vivo* du glutathion sur le métabolisme auxinique permettrait de comprendre, dans une certaine mesure, le rôle joué par ce corps sur l'activation de la croissance des néoformations (9).

Essais 2. Effet *in vitro* du glutathion sur la destruction auxinique

A 2 ml d'extrait (préparé à partir de néoformations développées sur des tissus cultivés en milieu nutritif habituel, dépourvus de glutathion), on ajoute 4 ml de la solution tampon à pH de 6,1 et au temps 0 un mélange préparé immédiatement de 2 ml d'ABIA et de 2 ml de glutathion. Après une période d'incubation variable (obscurité, dans un agitateur (8 oscillations/5 sec) thermostat (22,0°C \pm 0,5), on prélève 2 ml du mélange précédent qu'on ajoute à 8 ml du réactif. Les résultats (compte tenu des essais à 0, des essais témoins, de la dégradation spontanée de l'ABIA et de l'action directe du glutathion sur l'inactivation non enzymatique de l'ABIA) sont reportés dans la Figure 2.

Ces données nous montrent que le glutathion assure un ralentissement de la destruction *in vitro* de l'ABIA, inhibition d'autant plus nette que la concentration de ce corps est plus faible.

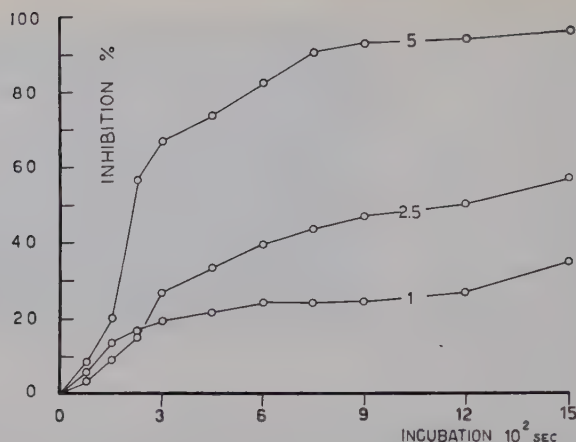


Figure 2. *Effet in vitro du glutathion sur la destruction auxinique.* On a reporté en fonction de la durée d'incubation le % d'inhibition de l'activité auxines-oxydasique. Ces % ont été calculés en tenant compte de la variation de l'activité enzymatique (lot témoin) en fonction de la durée d'incubation. Si le % est élevé, cela signifie que l'activité des auxines-oxydases est fortement ralentie, donc que la destruction des auxines est faible.

Les essais ont porté sur les concentrations suivantes de glutathion: 1, 2.5 et 5 µg/ml.

Discussion

Ainsi le glutathion assure une inhibition, *in vivo* et *in vitro*, de l'activité auxines-oxydasique des néoformations de tissus cultivés *in vitro*. Cette substance empêche donc une destruction rapide des auxines endogènes et favorise ainsi l'accumulation des hormones de croissance dans les tissus traités. Ces résultats sont à rapprocher des observations que nous rapportons récemment ici même (8). Nous montrions en effet que le long de l'axe radiculaire, la teneur en auxines et l'activité auxines-oxydasique étaient loin d'être identiques. Au-niveau du méristème par exemple, la concentration en auxines était très élevée et l'activité auxines-oxydasique, au-contre, très faible. Cette accumulation d'hormones devait être précisément attribuée au fait du peu d'activité des enzymes y relatives. La détermination de la teneur en fonction SH, dans les mêmes conditions, devait nous permettre de mettre en évidence que les corps sulfhydrylés étaient particulièrement abondants dans le méristème. Nous avons conclu alors que les composés à fonction thiol devaient être vraisemblablement des inhibiteurs auxines-oxydasiques. Dans le présent travail, les faits rapportés à propos du glutathion, un des composés sulfhydrylés type, confirment notre hypothèse antérieure basée sur l'analyse de phénomènes internes. Des relations paraissent ainsi exister sûrement entre les processus biochimiques caractérisant l'évolution métabolique des auxines et le rôle joué

par les composés à SH dans les tissus vivants. Il est toutefois certain que le glutathion en particulier, contrôlant d'autres mécanismes, comme par exemple les prénomènes d'oxydo-réduction, peut aussi être impliqué, mais par une voie différente, dans les cycles de transformations enzymatique des hormones de croissance.

Summary

Glutathione applied at a concentration of 1.10^{-7} to 1.10^{-4} M (added to Heller's basic plant tissue nutrient) produce in vivo a decrease in IAA-oxidase activity in young cells of in vitro carrots cultures. This inhibition of IAA destruction was also obtained in vitro on the same material. Consequently, the accumulation of auxins appears to be closely associated with increase of SH compounds concentration.

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Interaction of Gibberellic Acid and Vernalization in the Dwarf Telephone Pea

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Introduction

Varieties of the pea (*Pisum sativum* L.) have not generally been recognized as being naturally vernalizable (Highkin 1956). However, Highkin has reported that the two late-flowering varieties Unica and Zelka may be vernalized and that the effect may be manifested in the node of flowering as well as in vegetative growth. The tall Telephone pea, another late variety, is also vernalizable (Went 1957).

Leopold and Guernsey (1954) reported that the application to Alaska pea seeds of low concentrations of naphthaleneacetic acid combined with a brief cold treatment may effect a decrease in the number of nodes to the first flower. The authors state that a treatment of seeds or seedlings with auxin followed by a brief low-temperature treatment is promotive of flowering in plants generally.

Brian and Hemming (1955) have reported several effects of gibberellin upon growth and development of normal and dwarf varieties of peas. The most conspicuous of the gibberellin effects on pea growth is rapid internodal elongation, according to these authors. Gibberellin does not usually change the number of internodes in treated plants (Stowe and Yamaki 1957). Wittwer *et al.* (1957) studied the effects of gibberellin on the flowering of beans, tomatoes, and lettuce. Earlier flowering in these plants resulted from an acceleration of vegetative growth, and the number of internodes was not altered.

The present authors have found (Bonde and Moore 1958) that the application of gibberellic acid (GA) increases the number of nodes to the first flower in the dwarf Telephone pea but not in the tall Telephone variety. Since cold treatment (vernalization) of other pea varieties causes the plants to flower at a lower node, while gibberellin may have the opposite effect in the dwarf Telephone variety, *i.e.*, increase the number of nodes to flower, it seemed of interest to test the interaction of GA and vernalization in this plant.

Modifying the terminology of Highkin (1956), an inductive effect of cold treatment on flowering alone will be referred to as *reproductive vernalization*. Highkin's term *vegetative vernalization* will be used to designate the distinct inductive effect of cold treatment on vegetative development.

Materials and Methods

Seeds of the dwarf variety of the Telephone pea were used in the present study. The gibberellic acid (GA) used was in the form of the potassium salt. Stock solutions were made by dissolving 7.5 mg. of the pure potassium salt in 5 ml. of ethanol and adding this solution to 500 ml. of distilled water. The desired concentration of 1.5 mg./l. was made by diluting the stock solution with distilled water to proper volume.

In the vernalization procedure cylinders of a diameter slightly greater than that of a fully imbibed Telephone pea seed were made of paper. Twenty such cylinders were placed in each of six 400 ml. beakers and cut to the same height as the beakers. They were then filled to within approximately one inch of the top with fine-grade vermiculite, and the beakers filled with cylinders and an extra supply of vermiculite were autoclaved. One imbibed pea seed was then planted in each cylinder and covered with sterile vermiculite. The vermiculite was moistened with complete nutrient solution, and more solution was added as needed during the vernalization period. The cylinders were then placed for periods of 10, 20, or 30 days in a refrigerator maintained at 4°—5°C.

Seeds treated with GA before vernalization were soaked in a GA solution of 1.5 mg./l. for six hours at 20°C, while the seeds to be vernalized without previous treatment with GA were soaked in distilled water for six hours at 20°C before the beginning of the vernalization period. One lot of unvernallized seeds was planted directly in soil in the greenhouse after imbibition in water and one after imbibition in GA solution.

At the end of the vernalization period the treated seedlings were transplanted into soil-filled flats in the greenhouse. Except with the lots vernalized for 30 days, the paper cylinders were left intact when the seedlings were transplanted. Seedlings treated with GA after vernalization were thoroughly wetted with a single aqueous spray of GA nine days after removal from the cold. Tween 20 was added as a wetting agent in the aqueous sprays.

With the exception of the time spent in the vernalization process, all plants were grown in a greenhouse under long-day conditions (16 hours of light per 24 hours). The temperature was maintained in a range of 18°—22°C. Nutrient solution was applied to the soil in each flat approximately every two weeks.

Results

The percentage survival of vernalized seedlings was quite low in several cases (see Table 1). Despite efforts to prevent contamination, mold attacked some of the soaked seeds in the refrigerator and prevented germination of some of the original twenty seeds in each group.

At the end of the vernalization period the radicles of the seedlings vernalized 10 days were approximately 0.8 to 1.0 cm. long. Those of the seedlings vernalized for 20 days were approximately 1.5 to 3.5 cm long, and those of the seedlings vernalized 30 days were approximately 4.5 to 5.0 cm. long. Only in the seedlings vernalized for 30 days had the epicotyls clearly emerged from the seed coats at the time of removal from the cold. There was no apparent injury to the epicotyls or any other parts of these seedlings.

After the seedlings were removed from the cold and transplanted into the greenhouse, they began to grow very vigorously. It is evident from the data that the longer the time the seedlings were in the cold, the more rapid was the rate of growth after removal from the cold, since the vernalized plants were as large as or larger than the unvernallized plants even though they had been highly retarded in growth for 10, 20, or 30 days during the vernalization period.

Plants sprayed with GA as early seedlings or in more advanced stages respond to the acid with spindly stem growth, greatly increased stem elongation, and chlorosis, especially in the initial phase of the response. There is a

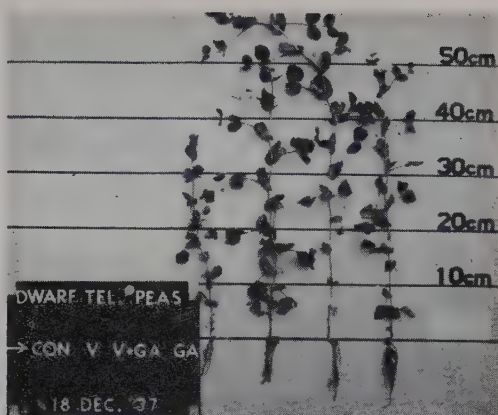
Table 1. *Comparison of vegetative growth of dwarf Telephone peas after various treatments.*
Average data are presented.

Treatment	No Plants in Lot	Shoot Length cm.	Whole Plant		Leaf ¹		
			Fresh Weight gm.	Dry Weight gm.	Leaflet Width cm.	Stipule Width cm.	Petiole Length cm.
No Cold; No GA (Control)	20	42.1	6.1	0.61	2.5	2.3	4.0
GA; No Cold	4	51.6	12.1	1.15	3.2	2.8	5.1
Cold 10 Days; No GA...	9	51.5	10.2	0.94	3.3	3.1	5.3
GA; Cold 10 Days.....	8	57.8	12.2	1.16	3.3	3.1	5.6
Cold 10 Days; GA	10	68.8	10.4	1.00	3.3	2.7	4.7
Cold 20 Days; No GA...	9	39.8	7.1	0.68	2.8	2.2	4.0
GA; Cold 20 Days.....	6	35.2	6.4	0.48	3.0	2.4	4.6
Cold 20 Days; GA	13	56.2	6.3	0.63	2.8	2.1	4.4
Cold ² 30 Days; No GA	4	48.6	7.4	0.83	3.0	2.8	4.8
GA; Cold 30 Days	4	37.8	5.4	0.61	2.8	2.4	4.3
Cold 30 Days; GA	6	51.7	6.6	0.79	2.6	2.4	4.4

¹ One leaf at the tenth node was measured on at least 4 plants in each lot.

² The plants vernalized 30 days were harvested 13 days later than the other plants.

Figure 1. *Contrasting growth form of plants subjected to various treatments. On the extreme left is shown an untreated control plant. Next, proceeding to the right, is a plant vernalized for 10 days with no GA treatment. The third plant was vernalized for 10 days and was sprayed with GA 9 days after removal from the cold. The fourth plant was sprayed with GA at the same concentration but did not receive cold treatment.*



lack of positive evidence that GA induces increase in leaf size in dwarf peas. In sharp contrast to those treated with GA, plants which received only vernalization treatment were very sturdy in growth. The stems were thick and succulent, and the leaflet blades were much enlarged over those of the control plants. Plants treated with GA in addition to vernalization showed still greater growth. Leaflet width and stipule width at the tenth node were also increased by GA treatment before vernalization. Vernalization alone increased leaf growth, but the response was enhanced by GA before vernalization.

Growth after vernalization was very rapid, and the rate increased with the time of vernalization, as is shown by the fact that plants which received vernalization for 30 days without GA were taller than the controls when harvested. This fact becomes more meaningful when it is realized that while the controls were harvested 13 days earlier than the plants vernalized 30 days, the latter were kept for the first 30 days of their growth period at a temperature of 4°—5°C.

The control lots and the lots vernalized for 10 and 20 days were harvested 71 days after planting. The three lots vernalized for 30 days were harvested 13 days later, or 84 days after planting. The data in Table 1 were taken at the time of harvesting.

Figure 1 shows the contrasting growth form of plants subjected to various treatments. The plant on the extreme right in the photograph is one week older than the other plants shown, but the other three plants are of the same age. Despite the greater age of the fourth plant, it is only slightly taller than the second plant, which was vernalized 10 days.

At the time of harvesting the number of nodes to the first flower was counted on all plants, beginning with the cotyledonary node. There was no

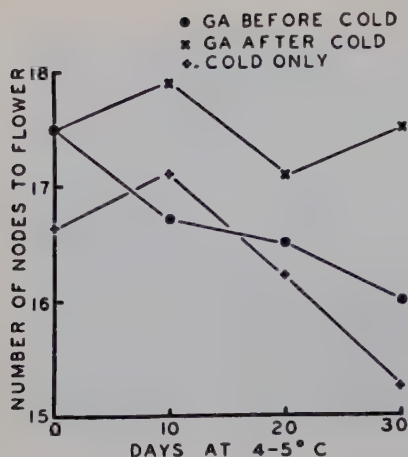


Figure 2. *Interaction of GA and vernalization in affecting the number of nodes to the first flower in dwarf Telephone peas. The cotyledonary node was taken as the first node.*

significant difference in the time required to flower in these plants, except in the case of the group vernalized for 30 days. The group vernalized 10 days flowered essentially at the same time as the controls, and the group vernalized 20 days flowered two or three days later. The group vernalized 30 days did not begin to flower until some thirteen days following the initial date for the group vernalized 20 days. The group soaked in GA solution before planting flowered at the same time as the group soaked in distilled water. All flowers in these plants were axillary and solitary.

Ten days was not sufficient to produce a reproductive vernalization effect in the dwarf Telephone pea. If height to the first flowering node is used as a measure of vegetative vernalization, then there was effective vegetative vernalization by the three treatments used on the 10-day plants. In Table 1 shoot length corresponds to height to the first flower, since at harvesting little or no shoot had developed above the flower. Cold treatment alone for 10 days induced stem growth as compared to the control plants, and GA before or after cold treatment increased this growth still more. Vegetative vernalization by cold treatment alone, however, was not effective with periods of 20 or 30 days. GA after cold treatment for periods of 20 and 30 days did increase stem growth, while GA before the cold treatment did not.

The vernalization effect on the node of flowering is shown by the data in Figure 2. Ten days of cold treatment without GA did not decrease the number of nodes to the first flower. In fact, a slight increase in the number of nodes to flower was noted. Twenty and 30 days cold treatment did significantly decrease the number of nodes to flower, the decrease being greater with the longer time of vernalization. However, the partial blocking of the reprod-

active vernalization effect on the plants treated with GA before 20 and 30 days of cold treatment is evident. Gibberellic acid treatment after 10, 20, and 30 days cold treatment was not only effective in completely devernializing the plants as regards the flowering node, but also in increasing the number of nodes to flower in all three groups as compared to the control plants.

Discussion

The chemical vernalization of Alaska peas by naphthaleneacetic acid and cold treatment reported by Leopold and Guernsey (1954) is especially interesting in the present connection. With the use of naphthaleneacetic acid combined with cold treatment, the number of nodes to the first flower was decreased, and thus *chemical vernalization* was effected. In contrast, the present authors have found that GA after cold treatment increases the number of nodes to the first flower in the dwarf Telephone pea over the number without GA, and thus a *chemical devernialization* has been effected. Further, GA treatment before cold treatments of 20 or 30 days partially prevents vernalization.

The results described in this paper indicate another GA effect on plant growth and development which is different from the effect of typical auxins. The term *auxin* is a generic term for compounds having the capacity to induce elongation in shoot cells (Tukey *et al.*, 1954). Gibberellic acid does have this essential capacity. Whether the removal of limitations on cell elongation by GA is by suppression of an inhibitor, synergism, or inherent stimulation remains uncertain. On the basis of the present investigation, it appears that GA may inhibit the vernalization effect on flowering while stimulating shoot-cell elongation at the same time.

Devernialization by heat following cold treatment has been known for many species for years. Highkin (1956) reported that heat treatment completely reverses reproductive vernalization in the pea variety Unica, but that heat only partially reverses vegetative vernalization. Similar results were reported for the variety Zelka. It appears that GA produces the same qualitative effect as heat treatment in the devernialization process. Highkin also reported that pretreatments of seeds of the two pea varieties for more than three days at 20°C and more than one day at 26°C prevents vernalization, and here, too, the effects of heat and GA with respect to prevention or reversal of the vernalization effect on flowering are similar. In the present experiments seeds were subjected to pretreatment with GA for only six hours before vernalization, but the GA was subsequently present inside the seeds after having been imbibed.

Of still further interest are the reports of Lang (1956, 1957) and Carr *et al.* (1957) that GA can replace the cold requirement in a number of cold-requiring plants (*e.g.* *Hyoscyamus niger* and *Centaureum minus*). Harrington *et al.* (1957) reported that gibberellins induced non-vernalized endive plants to flower. In the present study GA was shown to interfere with the cold effect on flowering in the dwarf Telephone pea.

Summary

1. Dwarf Telephone peas were *vegetatively vernalized* by cold treatment with and without GA for a period of 10 days. Cold treatments of 20 and 30 days were effective in promoting vegetative growth only when followed by GA spray, the response of plants receiving GA before the cold treatment or cold treatment without GA was much less.
2. *Reproductive vernalization* of the plants was accomplished with cold treatments of 20 and 30 days but not with 10 days. GA treatment before vernalization of 20 and 30 days duration partially blocked the reproductive vernalization effect. GA treatment after vernalization of the same durations completely reversed the reproductive vernalization effect. A *chemical devernalization* was thus effected.

The authors wish to express their gratitude to Merck & Co., Inc., Rahway, New Jersey, for supplying the sample of gibberellic acid used in this study.

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Evidence for Separate Mechanisms of Sodium and Potassium Regulation in *Hormosira banksii*

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Introduction

In New Zealand the fuclean alga *Hormosira banksii* occupies a position in the lower part of the littoral zone, so that it is subject to drying and change of osmotic pressure. It has been found that the alga forms excellent material for physiological investigations because of its bead-like morphology. The thallus consists of a series of hollow bladders united to each other by a solid mass of nodal tissue, and cutting through the node does not appear to interfere with individual bladder metabolism. In the present investigation, which forms part of a more extensive study, use has been made of the alga to study movement of sodium and potassium ions.

Experiments on the littoral marine alga *Ulva lactuca* (Chlorophyceae) have been described by Scott and Hayward (1953, 1954, 1955) who suggested that two independent metabolic mechanisms are operative in moving sodium out and potassium into the cells against their respective electrochemical gradients. Blinks (1951) claims that the osmotic pressure of algal cell sap is a result of inorganic salts, and intertidal dehydration causes negligible injury if these salts can enter as fast as external sea-water concentration occurs. He found that the K^+ concentration of the cell sap of *Valonia* rose by 30 % when the surrounding sea water evaporated, but could not decide whether the mechanism of accumulation suggested by Osterhout (1931) or if nitrogenous or phosphorylation mechanisms were involved.

Hormosira shows extreme tolerance to water loss, partly due to the unique 'chain-of-bladders' morphology, each vesicle containing its own water reservoir. The writer has demonstrated an increase of salt concentration within the bladders on dehydration that is less than that calculated from the amount of water lost — *e.g.* at 20 % dehydration, the calculated value is for a chlorinity of 0.73N, but the observed value is 0.69N. Since dehydration initially results in an increased rate of oxygen uptake, it is of interest to examine the possibility that this may be the result of an alteration of cation regulation in the tissue. The purpose of this communication is to report the effect of simulating various field conditions, and also the effects of various inhibitors on the sodium and potassium balances, as revealed by tissue analyses.

Methods

After at least 12 hours washing and aeration following collection, large bladders of Hormosira were selected, and in the case of the dehydration experiments injected with appropriately modified sea water. In the accumulation experiments in liquid, the ends of each bladder were sliced off in order to give a hollow cylinder of tissue. At the conclusion of the experiment, each bladder was sliced longitudinally into four equal portions: one portion was discarded, the other three were distributed, one to each of three treatments ('killed', 'long dip', 'quick dip' — see below). Each set of tissue was delicately blotted and the fresh weight determined. The samples were wet ashed with 1 ml. of concentrated A.R. HNO_3 , made up to volume, and filtered through Whatmans No. 40 'ashless' paper before analysis for Na^+ and K^+ using a standard 'EEL' flame photometer. The transmission values were compared with those of known standards containing the same proportion of acid as the samples of the extracts.

Stock solutions of inhibitors were adjusted to the appropriate pH before addition to flasks containing the tissue. All experiments in air were conducted at room temperature (22°C), and in liquid, at 30°C in a thermostatically controlled water bath. All determinations were made in triplicate.

Scott and Hayward (1954), concerned lest contamination by sea water retained in the extracellular spaces would affect their electrolyte analyses, devised an elegant technique of dipping the tissue into isotonic sucrose followed by blotting with absorbent tissue. They found in a series of tests that all the extracellular and none of the intracellular cations were removed, and hence they claim their data represents the true cellular sodium and potassium concentrations. The total cellular sodium and potassium, however, comprises both bound cations (*i.e.* structural, or bound to cell membranes) and cations actively accumulated (*i.e.* held in the vacuole, as well as cytoplasmic sodium and potassium). Furthermore, no separate account is taken of cations present in the free space of the tissue, the nature of which may alter with different experimental treatments.

It was found that a 3 second rinse ('quick dip') in isotonic sucrose removed the superficial seawater from Hormosira tissue, but no electrolyte was lost from the

free space since there is no appreciable difference between tissue samples that have been removed after 1, 3, 5 and 10 seconds, but there is a steady loss after this period. If the tissue is analysed for the cations, then we obtain the *total* Na^+ and K^+ of the sample. Furthermore, using Eppley and Blinks' (1957) method of free-space and cell-space measurement it was determined that the free space of *Hormosira* reaches equilibrium with the external solution within one hour. When portions of tissue are soaked for 60 minutes in a large volume of isotonic sucrose ('long dip'), lightly blotted, and analysed, the data provides estimation of the total cellular Na^+ and K^+ . If portions of tissue are killed by boiling or by cold ethyl alcohol ('killed'), thoroughly washed and soaked in sucrose as above, the Na^+ and K^+ concentrations relate to the *structurally bound* cations. Hence subtraction of 'long dip' from 'quick dip' values gives the free space Na^+ and K^+ , and subtraction of 'killed' from 'long dip' values gives the cytoplasmic cation component. By this procedure, the ionic constituents of the tissue can be separated into three fractions, and hence variable amounts of adsorbed ions (*i.e.* non-exchangeable) will not affect the results pertaining to the free space and cytoplasmic concentrations.

Results

Tissue under dehydration

In experiments on respiratory oxygen uptake following water loss, bladders were dehydrated by 20 %, 40 % and 60 % of the tissue water content (Bergquist 1957). The same dehydration levels were used in these experiments. As shown in figure 1, there is a progressive increase of free space Na^+ and K^+ — the latter being very marked at the 60 % level. Cytoplasmic cation concentration also increases, but the behaviour of the bound fraction is not regarded as significant and is probably purely fortuitous.

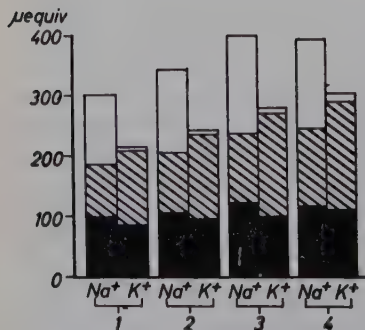
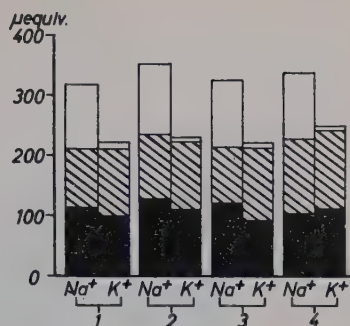


Figure 1. Amount of Na^+ and K^+ in various tissue components after dehydration, 1 — fully hydrated. 2 — 20 % dehydrated; 3 — 40 % dehydrated; 4 — 60 % dehydrated; Tris (hydroxymethyl)-aminomethane-buffered (Tris) natural sea water was injected into bladder cavities. To eliminate effects introduced by simultaneous photosynthetic activity, the experiment was performed in the dark using a gentle air-stream from a hair-drier so that the period to maximum dehydration (60 % of water lost) was the same as experienced in the field. Black — structurally bound component; hatched — cytoplasmic component; plain — free space component. On the ordinate $\mu\text{equiv/g. fresh weight}$.

Figure 2. Amount of Na^+ and K^+ in the various tissue components on reimmersion following dehydration. 1 — tissue previously fully hydrated; 2 — tissue previously 20 % dehydrated; 3 — 40 % dehydrated; 4 — 60 % dehydrated. Tissue was reimmersed after dehydration, each sample in 500 ml. of sea water in the dark for 6 hours at 20°C. Diacritic symbols as in Figure 1.



Tissue on reimmersion

If tissue is reimmersed after dehydration, there is a return of the previously accumulated ions back to the initial distribution (Figure 2). Assuming that the fully hydrated tissue has neither accumulated nor lost ions over the experimental period, the degree of return of the previously accumulated ions can be expressed as a percentage (Table 1).

Thus there appears to be little retention of the ions accumulated during the period of dehydration, save for Na^+ ions in the tissue dehydrated to 60 % — and at this dehydration level there is no respiratory recovery on reimmersion comparable to that shown at the other dehydration levels (Bergquist 1957).

Tissue reimmersed with 2,4-dinitrophenol added internally

The comparison provided by this data (Figure 3) with that above is particularly striking. In every case there is an increase in free space, cytoplasmic, and bound Na^+ , and also in every case there is a decrease of cytoplasmic K^+ , although bound and free space K^+ remain essentially constant. Scott and Hayward (1955) also report an increase of cellular Na^+ and a decrease of cellular K^+ on the addition of 4,6-dinitro-*o*-cresol although over a much longer time period. This suggests that not only does active

Table 1. Percentage return of Na^+ and K^+ to previous levels following dehydration.

% dehydration	Na^+	K^+
0	100.0	100.0
20	92.8	98.6
40	103.6	103.4
60	82.7	102.8

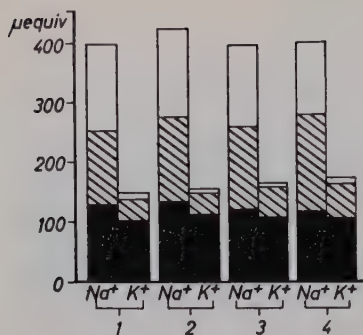


Figure 3. Amount of Na^+ and K^+ in the various tissue components after 2,4-DNP has been added to previously dehydrated bladders. 1 — tissue previously fully hydrated; 2 — tissue previously 20 % dehydrated; 3 — 40 % dehydrated; 4 — 60 % dehydrated. After dehydration, internal sea-water withdrawn and replaced by Tris-buffered sea water containing $2.33 \times 10^{-4} M$ DNP (pH 7.5 — this concentration of DNP induces maximum stimulation of oxygen uptake at this pH). Bladders were then placed in 500 mls. Tris-buffered sea-water and left in the dark for 6 hours at 20°C with continuous aeration. Diacritic symbols as in Figure 1.

transport stop after the application of DNP, but also that K^+ ions leak out and Na^+ ions flow into the cytoplasm in accordance with the electrochemical potential gradient. It would appear that membrane permeability has been affected as well as the phosphate bond energy-demanding processes of cation regulation.

Effect of iodoacetate

Scott and Hayward (1953) established a marked loss of potassium and gain of sodium when iodoacetate was added to tissue of *Ulva* kept in the dark, although the inhibitor was ineffectual in the light. Pyruvate was found to protect the tissue from Na^+ increase caused by the inhibitor, whereas phosphoglycerate protected against K^+ loss. They concluded that inhibition of 3-phosphoglyceric acid dehydrogenase prevented K^+ accumulation but that the effect on the sodium-regulating mechanism was more indirect.

Scott and Hayward performed their experiments with iodoacetate of pH 7.5. The pK of iodoacetic acid is 3.1, and since Simon and Beevers (1952) state that the activity of an inhibitor is virtually independent of pH below the pK, but diminishes above it, experiments have been carried out at pH 4.8 as well as at pH 7.5. Allen's artificial sea water (Allen 1914) was used with reduced Ca and Mg contents to enable the NaH_2PO_4 of the citrate buffer to be mixed without precipitation. Potassium ions of the usual phosphate buffers stimulate both the respiration rate and the uptake of K^+ , but despite being a metabolic intermediate, citrate causes no detectable respiratory stimulation or enhanced uptake of cations. The K^+ concentration of the mixture was 50 mequiv/litre. A 6 hours pretreatment period (Turner 1938) was given to tissue placed in flasks and continuously aerated in the dark. Exogenous pyruvate at 10^{-2} and $10^{-3} M$ was added to circumvent the block at the level of 3-phosphoglyceraldehyde.

Parallel gas exchange experiments were performed using standard Warburg manometry at 30°C .

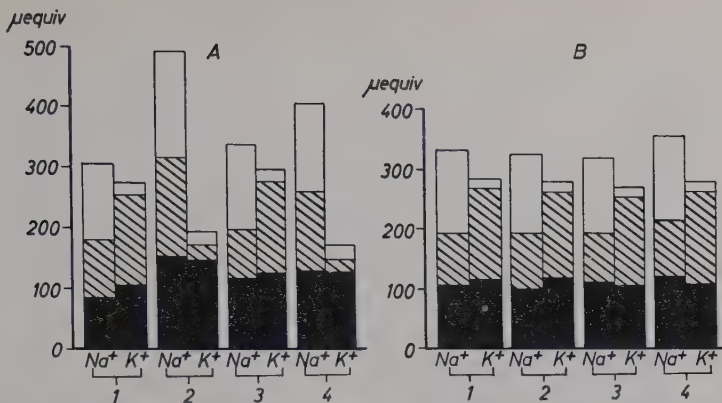


Figure 4. Amount of Na⁺ and K⁺ in the tissue fractions of bladders exposed to iodoacetate. A. At pH 4.8: 1 — control; 2 — plus iodoacetate; 3 — plus pyruvate; 4 — plus iodoacetate and pyruvate. B. At pH 7.5: 1 — control; 2 — plus iodoacetate; 3 — plus pyruvate; 4 — plus iodoacetate and pyruvate. Diacritic symbols as in Figure 1.

As shown in Figure 4 A, when iodoacetate is added at pH 4.8, there is a marked increase of Na⁺ in all cell fractions, but the effect on K⁺ is to increase slightly the free space value but greatly decrease the cytoplasmic portion. In the experiments at pH 7.5, there is little significant difference between treatments (Figure 4 B). It is also evident that pyruvate does not protect the tissue from the influence of iodoacetate at pH 4.8.

From the oxygen uptake data (Figures 5 A and 5 B) it is evident that when iodoacetate is added at pH 4.8 there is a progressive decline that is not prevented by pyruvate, and the tissue is apparently dead. This effect was observed at iodoacetate concentrations of 10^{-3} , 10^{-4} , and $10^{-5}M$, and pyruvate at 10^{-2} or $10^{-3}M$ provided no protection. Oxygen consumption of tissue exposed to iodoacetate and iodoacetate plus pyruvate at pH 7.5 was as rapid as, or even above the control value. Data for the control at pH 4.8 showed a slight depression of oxygen uptake but addition of pyruvate ($10^{-2}M$) allowed a slight increase.

The important information provided by this data is the vulnerability of the tissue to iodoacetate at low pH and the essential irreversibility of this effect, since well washed bladders previously exposed to the inhibitor also showed no recovery, and furthermore the inhibitor is completely ineffectual at the higher pH.

One is thus led to conclude that for *Hormosira*, iodoacetate inhibition is essentially irreversible over the range of concentrations investigated at pH 4.8. Pyruvate does not allow continuation of metabolism in spite of this inhibition. It would appear likely that the iodoacetate is poisoning other sulfhydryl groups in the tissue, as well as the alkylation of the thiol groups of triose phosphate dehydrogenase.

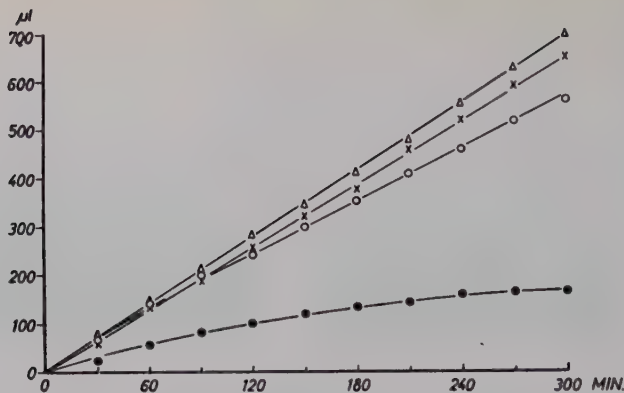


Fig. 5 A.

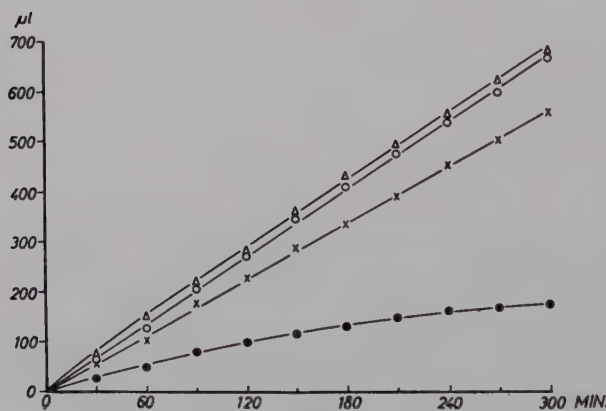


Fig. 5 B.

Figure 5 A and B. Oxygen uptake of *Hormosira* tissue exposed to $10^{-4} M$ iodoacetate and $10^{-2} M$ pyruvate. Suspending medium was citrate-buffered artificial seawater. Diacritic symbols: — A — \circ — control, pH 4.8; \bullet — plus iodoacetate, pH 4.8; \triangle — plus iodoacetate, pH 7.5; \times — plus pyruvate, pH 7.5. B — \circ control, pH 7.5; \bullet — plus iodoacetate and pyruvate, pH 4.8; \triangle — plus iodoacetate and pyruvate, pH 7.5; \times — plus pyruvate, pH 4.8. On the ordinate μl O_2 /g. fresh weight.

Effects of other inhibitors

Tissue exposed to $10^{-4} M$ cyanide shows a 50 % inhibition of oxygen uptake, while there is only a 20 % inhibition in the presence of the mercaptide-former, *p*-chloromercuribenzoate (PCMB), despite its more rapid reaction and great affinity for -SH groups unreacted upon by other alkylating reagents. To evaluate the effects of these reagents on cation distribution, $10^{-4} M$ KCN (pH 8.0) was applied to tissue bathed in normal Allen's sea water and modified sea water ($K^+ = 50 \mu\text{equiv/ml.}$), PCMB ($10^{-4} M$) at pH 7.5, as well as $2.33 \times 10^{-4} M$ DNP (pH 7.5).

Table 2. *Effect of various inhibitors on cation distribution in tissue of Hormosira. Data as % of appropriate control. FS=free space.*

Conditions	Fraction	Na ⁺	K ⁺
+KCN, pH 8.0	FS	204.8	97.6
modified sea water	Cytoplasm	199.4	98.8
K ⁺ =50 equiv./ml., Na ⁺ =550 µequiv./ml.	Bound	147.0	102.0
+KCN, pH 8.0	FS	173.5	107.0
natural sea water	Cytoplasm	203.5	96.6
K ⁺ =12 µequiv./ml.	Bound	122.4	108.4
+DNP, pH 7.5	FS	117.6	97.0
modified sea water	Cytoplasm	182.8	42.0
	Bound	134.2	92.2
+PCMB, pH 7.5	FS	62.0	85.9
modified sea water	Cytoplasm	202.0	104.4
	Bound	149.1	95.8

The unusual values for the free space Na⁺ in the presence of cyanide when tissue is exposed to increased salt concentration is shown in Table 2, which also shows that bound and cytoplasmic Na⁺ have increased (Table 2). The cytoplasmic Na⁺ has doubled in the presence of cyanide, DNP, and PCMB. With DNP the free space Na⁺ has increased slightly but has decreased with PCMB. The values for K⁺ show that only when tissue is exposed to DNP is there any marked variation and then only in the cytoplasmic fraction, the distribution being similar to the reimmersion values. There is about a 15 % decrease of free space K⁺ with PCMB (Table 2).

Discussion

Robertson (1957) has outlined the difficulties of using cation uptake in the measurement of apparent free space, and while the method described here is free to a certain extent from some of the problems raised, it cannot legitimately be used as a 'true' free space estimate. The values suggest that the method allows measurement of what Epstein (1955) terms 'outer space', since it is assumed that the concentration of ions removed by sucrose is the same as would be removed if those ions were allowed to diffuse out into an external solution that did not contain them. The 'outer space' should show no pH effect, but calculation from the data above shows that the free space increases with decreasing pH, and values from 27.1 to 48.3 % are higher than expected from data of isotope experiments (Bergquist, unpublished data).

This suggests that some fraction of the Donnan space has contributed to the ions removed by sucrose. It is interesting then, to speculate on the reasons for the increased value of the free space in the presence of cyanide. This free space alteration has also been confirmed by use of radioisotopes (Bergquist 1958). Simple cases of the occurrence of plasmolysis or Kappen-plasmolysis have been treated by Briggs and Robertson (1957) and a possible explanation, the exchange of bivalent cytoplasmic cations with predominantly univalent external solution cations with an increase of volume of both AFS and cytoplasm, does not explain the constant value for the free space and cytoplasmic K^+ . It may be that the inhibitor has altered to some degree the whole nature of the Donnan component, and this has had an effect on K^+ : Na^+ ratios, bound ion: free space ion ratios, and more particularly, on the ion-exchange portion of the tissue. Any one of these could be achieved by an effect on a labile binding mechanism that, after inhibition, allows the Na^+ to be easily removed from the system by sucrose, or, since the tissue is high in K^+ , alteration of the ion exchange portion of the system may not effect the K^+ but will effect the Na^+ ions.

It has been established by isotope measurements that the cell space of *Hormosira* is equivalent to 30.6 % of the tissue volume, a value that could be expected from microscopic examination of the very small cells with very thick walls. Calculation from the data cited above shows that the Na^+ concentration in the tissue is always below that of the external seawater (0.2—0.4M) while the K^+ concentration is well above (0.6—0.7 M). It would appear likely that sodium is continually passively diffusing into the cells and that there is a mechanism ('ion-pump') that is constantly expelling the ions.

A redox pump of the type suggested by Conway (1951, 1953), possibly located in the cytoplasm contiguous with the free space, would provide a suitable mechanism whereby electrons are transferred to oxygen, a cation is expelled, and an anion would pass out 'passively' to maintain electrical neutrality. Such interpretation is in agreement with the inhibitor data, where poisoning of the enzyme-system results in an increased cytoplasmic Na^+ concentration. Operation of a Na^+ expulsion 'pump' is also indicated by the experiments on the effect of dehydration and reimmersion, where cytoplasmic Na^+ accumulates as drying proceeds, possibly since the mechanism is working at full capacity when the plant is in sea-water and increased Na^+ concentration results in accumulation, since the 'pump' cannot cope with the situation at a sufficiently rapid rate. When the alga is returned to water, the rate of output remains high until the excess Na^+ ions have been expelled. If DNP is added after dehydration, the pump is uncoupled and Na^+ ions leak 'uncontrolled' into the cytoplasm.

The most impressive feature of the K^+ data is the complete indifference to all inhibitors used except the uncoupling agent, DNP. Even when the membrane component of the free space apparently suffers alteration with the addition of KCN, there is little effect on the K^+ concentration and the mechanism transporting the cation may possibly be considered as located in the vacuolar membrane. Such a suggestion is in accordance with the results observed on reimmersion when DNP is injected into the bladders and there follows rapid loss of cellular potassium. It would be possible for a shuttle-type of carrier, as suggested by Polissar (1954), involving cyanide-resistant enzymes to be in operation in K^+ regulation.

A mechanism of this type will allow K^+ ions to be accumulated when tissue is exposed to higher salt concentrations than normal. There is, however, a further problem, because examination of the data on K^+ distribution after dehydration and reimmersion, shows that there is a loss of the K^+ ions almost back to the original level. The only suggestions that can be made are (i) that there may be a K^+ expulsion mechanism operating when the alga is emersed; (ii) the K^+ accumulation mechanism may be working at an increased rate to give a net increase of influx over outflux, since the expelled or diffusing ions cannot be removed any further than the free space and are hence constantly recirculated, or (iii) there may be an inhibition of the efflux mechanism when the salt concentration is high. With the evidence at present available, the writer prefers the second possibility.

Summary

1. A method is described whereby the cation concentration of the tissue of *Hormosira banksii* is separated into three components, allowing more accurate estimation of the effects of various treatments.

2. When tissue is dehydrated, there is an accumulation of both Na^+ and K^+ in the cytoplasm. On reimmersion the greater portion of the accumulated ions are expelled from the tissue. Addition of DNP allows equilibration by diffusion of the previously accumulated ions in accordance with their concentration gradients.

3. Iodoacetate in a variety of concentrations appears to be lethal to the tissue at pH 4.8 as judged by ion analyses and oxygen consumption. There is no effect at pH 7.5, and pyruvate does not allow metabolism at the lower pH.

4. Cyanide, PCMB, and DNP have a marked effect on the Na^+ distribution, but only DNP effects the K^+ concentration as compared with controls.

5. The method of free space estimation is discussed and it is thought that

it cannot be used as an estimate of the tissue apparent free space. Tentative explanations are advanced for the effect of KCN on the Na^+ space.

6. It is suggested that there are two mechanisms regulating cation distribution — a redox-type 'pump' controlling the Na^+ level, and possibly a mechanism similar to that suggested by Polissar (1954) regulating the K^+ level.

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Influence of Plasmolysis and Inhibitors on the Sucrose Inversion in Wheat

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Introduction

It has been shown in a previous paper that the splitting of sucrose in wheat roots takes place in the system usually designated as the free space (Burström 1957). There is some diversity of opinions regarding the nature of this concept: whether the space is confined to the cell wall only or also extends into the cytoplasm, and whether the computations carried out give a true estimate of its size or not (cf. Kylin och Hylmö 1957, Briggs and Robertson 1957, Levitt 1957). Since the sucrose hydrolysis up till now is the only metabolic reaction explicitly assumed to occur in the free space, a closer study of the reaction was found justified. Several enzyme reactions have been localized to the surface of cells or organs (cf. Rothstein 1956) but their cytological location is nevertheless uncertain. In a picture Rothstein located invertase and phosphatase on the outer cell wall surface, which means that the reactants should not even enter the free space. However the picture is certainly intended to give only a general idea of their location. Myrbäck (1957) also in rather loose terms placed the activity on the outside of the yeast "cell membrane" or in a region with the same pH as in the surrounding solution. The latter conclusion is based on the identity of the pH-curve for the enzyme *in situ* and in true solution. The validity of this argument has been doubted by McLaren (1957). It stands to reason, nevertheless, that any direct influence of the external pH on a reaction *in vivo* indicates its location

on the surface or in the free space, regardless of whether the pH-activity curves are identical or not. Putman and Hassid (1954) have indisputably shown that in Canna leaves invertase is located on the surface of the mesophyll cells. This is probably a general phenomenon in plant cells (cf. Burström 1957).

In wheat the splitting of sucrose was found to proceed at a rate exactly proportional to the root surface. This was interpreted as indicating an inversion in the free space on the root surface, only the high rate of this inversion preventing sucrose from passing into the interior of the root. This assumption can be verified in the following way. If the root is plasmolyzed, the pathways for the external solution into the root increases and this would then lead to an increased sucrose inversion, provided that the active surfaces were not injured by the plasmolysis. The present investigation confirms this assumption. In order to characterize the reaction, some inhibitors were also tested on both normal and plasmolyzed roots.

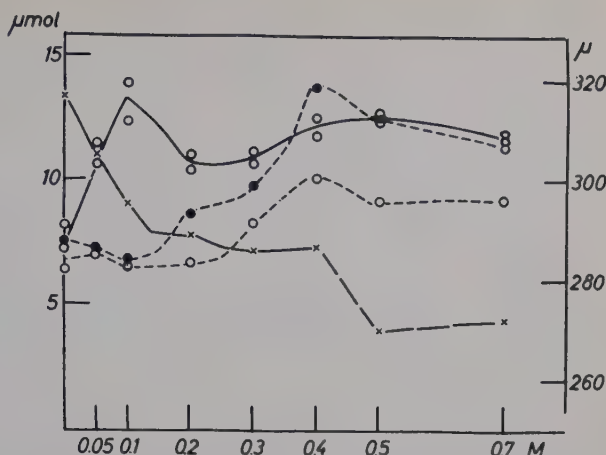
The paper, moreover, records experiments of the same kind with coleoptile sections. Such are extensively used in sucrose solutions for growth studies, and their reaction to sucrose is therefore of a special interest.

The methods employed are exactly the same as those described earlier (Burström 1957). The sucrose concentration was always 10^{-3} M and the experimental period 24 hours, the temperature 22°C. Mannitol was added as a plasmolyticum. It is theoretically non-reducing, but as most organic compounds it increases the blank reduction in the sugar determinations. The blank values were determined in every series of experiments and subtracted from the sample values. Even with the highest mannitol additions this correction amounted on an average to less than 5 per cent of the obtained inversion. Sterile filtering and standing of the solutions under the experimental conditions did not change the blank reduction. Roots increased the reduction of mannitol solutions in the absence of sucrose. The reason was not investigated, but it might have been caused by exudation of solutes. In 0.4 M mannitol it amounted to a value corresponding to c. 0.3 μ mol sucrose per 100 mm. roots, and in 0.7 M to 0.7 μ mol, or c. 3 and 6 per cent, respectively, of the obtained sucrose inversion. This error was not corrected.

Results with Roots

The influence of plasmolysis on wheat roots is depicted in Figure 1. The decrease in size of the tissues was determined on a large material by measuring the epidermal cell lengths (see the figure). There is a sharp break in the cell length curve at 0.13 M mannitol, which represents the point of incipient plasmolysis. Between 0.13 and 0.4 M the cell lengths remain constant, but above this concentration there is an irregular shrinkage under corrugation of the cell walls (broken curve in Figure 1). Thus the course of the plasmolysis seems to be of the normal, regular type.

Figure 1. *The influence of external mannitol additions on the splitting of sucrose. On the abscissa mannitol M , on the left ordinate sucrose hydrolyzed μmol per 100 mm. root length. \circ — without any further additions. \circ --- with CaCl_2 10^{-4} , \bullet --- with CaCl_2 $10^{-4} + \text{KNO}_3$ 10^{-3} . On the right ordinate cell lengths showing the contraction of the roots. \times — reduction in cell length with a break at 0.13 M mannitol representing incipient plasmolysis, \times — cells shrinking with corrugated walls.*



The full-drawn curve in Figure 1 shows the sucrose inversion in a phosphate buffer of pH c. 6, without other metals than K and Na present. It is composed of three parts. *In the range of hypotonic solutions the inversion increases about 100 per cent.* Apparently at the point of incipient plasmolysis, or as near this point as it is experimentally possible to come, *there is a sudden drop in the inversion, and in the plasmolyzed tissue it increases again.* This surprising action of hypotonic mannitol solutions as well as the drop at incipient plasmolysis and the ensuing increase, were encountered in every series of experiments (only averages of complete concentration series are recorded in the figure). An attempt to study the nature of this change in inversion was made by applying certain inhibitors.

Calcium and other divalent cations inhibit the inversion (Burström 1941, 1957). In series with mannitol $\text{Ca } 10^{-3} M$ *cuts off the whole hypotonic increase and the inversion curve shows a steady increase from the point of incipient plasmolysis onwards.* The irregular behaviour during the shrinkage in the highest concentrations can certainly be disregarded as due to the mentioned artifact.

There is no difference in hydrolysis between + and -Ca at mannitol=0 in Figure 1. This depends upon the fact that the inversion is computed per root length and not per unit surface, and that the root hair formation and thus the surface, in spite of all precautions, varies from one series to another (Burström 1957). In the material in Figure 1 different mannitol concentrations with one Ca level were run simultaneously, thus the different curves cannot be directly compared. It has been exemplified repeatedly in previous communications, and verified in a number of new experiments not recorded (cf. also Figure 2) that under strictly comparable conditions Ca invariably reduces the inversion also in the absence of mannitol.

Table 1. *The influence of different stimulators and inhibitors on the surface inversion of sucrose by wheat roots. Standard experimental conditions. pH 6.5—7.0. Inversion μmol sucrose per 100 mm. root length.*

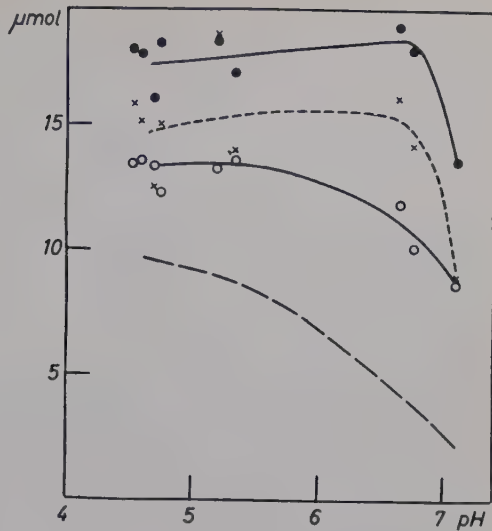
Substance added <i>M</i>	Na_2EDTA	2NaCl	Oxychinoline	HgCl_2	$\text{HgCl}_2 + \text{Mannitol}$ 0.4 <i>M</i>	$\text{UO}_2(\text{NO}_3)_2$
0	10.3	11.8 ± 0.3	10.0 ± 0.6	10.0 ± 0.8	13.8 ± 0.2	10.8
10^{-8}	×	×	×	10.9	13.6	×
10^{-7}	×	×	×	4.8 ± 1.2	13.3 ± 0.7	×
10^{-6}	×	×	10.5 ± 0.3	1.6	3.7	×
10^{-5}	12.5	12.1 ± 0.8	12.1 ± 0.6	1.4	×	10.0
10^{-4}	13.1	12.5 ± 0.4	11.6 ± 0.3	1.0	×	11.5
10^{-3}	16.9	10.5 ± 0.5	×	×	×	4.5

Ca is, of course, present also in roots not supplied with Ca. In order to remove these traces, experiments were carried out with additions of EDTA as the disodium salt (Table 1). The second column in the table shows a marked increase in inversion with increasing additions of Na_2EDTA . In order to eliminate the possibility of an action of Na, corresponding concentrations of NaCl were also tested (third column). It can be inferred that the complex-former causes an 80 per cent increase in sucrose inversion, presumably by removing an inhibiting divalent cation (Ca).

According to Martell (1957) the chelation of divalent cations by EDTA increases with increasing pH within the range 4.5 to 7, with a break at 7 owing to a change from di- to tri-basic dissociation of the acid. The pH-dependence of the EDTA-action on the sucrose inversion is depicted in Figure 2. Without addition of EDTA the usual pH-curve, rising towards pH 4, was obtained, and the "normal" shape from enzyme chemical studies (McLaren 1957) is still better represented in the presence of Ca 10^{-3} *M*. Removal of Ca and addition of EDTA promote the inversion particularly near pH 7 with a break in the EDTA action around that pH value, in agreement with the expectations. This confirms the assumption that EDTA acts by removing a divalent metal. The unexpected result is that *with decreasing Ca the pH-sensitivity of the inversion gradually disappears* within the whole range of pH 4.5 to 7, a phenomenon which does not seem to be common in enzyme reactions.

As a comparison with EDTA the heavy-metal chelating *oxychinoline* was tested (Table 1). It shows in physiologically reasonable concentrations no effect whatsoever. — Several metals have been employed as more or less specific inhibitors of saccharase. Rothstein and Hayes (1956) have especially studied UO_2^{+2} finding a blocking of probably carboxyl groups at high concentrations, leading to an inhibition of the hydrolysis. The living roots are little

Figure 2. *The relation between pH, addition of EDTA and sucrose splitting. On the ordinate sucrose hydrolyzed μmol . per 100 ml root length. \circ — without EDTA, \times --- EDTA 10^{-4} , \bullet — EDTA 10^{-3} M, — without EDTA + Ca 10^{-3} M (average curve).*



sensitive to uranyl ions, less so than to calcium. The reverse is obviously the case with yeast. It is difficult to infer that uranyl is a specific inhibitor of the root surface hydrolysis.

However, this may be the case with HgCl_2 (Table 1). In 10^{-7} M it causes over 50 per cent inhibition, which could be compared with, *e.g.*, the 10^{-4} to 10^{-3} M necessary for inhibiting the glucose uptake in rat diaphragm (Demis and Rothstein 1955). With mannitol present the sensitivity decreases to about one tenth. This can be explained in one of three ways. (1) The normal hydrolysis and that induced by mannitol are of different nature, and the latter is less sensitive to Hg^{+2} . It was assumed that the entrance of sucrose into the root was prevented by the surface inversion. If this is partly inhibited more sucrose could enter and compensate the lower inversion on the surface, but it should then be a mere chance that the inversion appears to be unaffected by 10^{-7} Hg, which in the absence of mannitol is strongly inhibiting. (2) The presence of mannitol or the changed osmotic conditions decrease the sensitivity to Hg^{+2} . (3) A further possibility is a decreased entrance of Hg or a decrease relative to the entrance of sucrose. These possibilities will be discussed below.

Results with Coleoptiles

Experiments were also carried out with the same technique on the sucrose inversion by sections of coleoptiles, giving consistently similar result. One example is presented in Table 2.

Table 2. *The sucrose inversion by coleoptile sections.* The same conditions as in the root experiments. pH 6.6—6.8. Initial length of sections 7.3 ± 0.1 mm. 5 sections per flask of 25 ml. solution.

Addition <i>M</i>	Final length of sections	Sucrose inverted	
		μmol per 10 sections	μmol per 100 mm section length ¹
None	13.6 ± 0.4	4.61	4.41
CaCl_2 10^{-3}	13.6 ± 0.4	1.80	1.72
Mannitol 0.5	7.7 ± 0.1	6.45	8.60
CaCl_2 + Mannitol	7.6 ± 0.2	2.51	3.37

¹ Average of initial and final length.

The aseptically raised coleoptiles were decapitated and sectioned in the way usually employed in growth studies. Since light was not avoided the sections grew during the time of experiment. Hence the inversion has been computed both per section and per length. The leaves were left in the coleoptile sections.

The inversion is not much slower than in roots, on an average $0.95 \mu\text{mol}/100 \text{ mm}^2$ and 24 hours against 1.88 in roots under standard conditions. The reaction to mannitol and Ca is the same as with roots, regardless of how the inversion is computed.

Insofar as coleoptile growth depends upon the uptake of sugar (Ordin, Applewhite and Bonner 1956), the hydrolysis of sucrose on the surface ought to be of some importance for the growth result. Coleoptiles like roots can be plasmolyzed in mannitol, and thus sucrose can not readily enter the cells either unless it is phosphorylated, whereas the uptake of hexoses ought to be easier. Since Ca inhibits the inversion strongly even in a low concentration and mannitol increases the same, they should affect coleoptile growth by changing the sucrose inversion, apart from other growth effects. Cooil and Bonner (1957) have actually found an inhibition of coleoptile growth by Ca. The $10^{-2} M$ Ca added in their experiments must cause a considerable inhibition of the inversion of the sucrose. — Generally, this phenomenon must be duly recognized if sucrose, or sucrose and mannitol together, are used for establishing controlled osmotic conditions in growth studies.

Discussion

In the presence of Ca the splitting of sucrose behaves as could be expected from previous results (Burström 1957). The reaction takes place on the root surface, that is in the free space on the surface of the root. The rate is so much higher than the diffusion of sucrose into the root that no sugar can

enter the interior. This condition prevails also in hypotonic solutions of mannitol. After plasmolysis, however, the rate of inversion increases. The reason should be that not only the transversal cell walls but also the cell cavities occupied by the plasmolyticum now serve as path-ways of the sucrose, facilitating the entrance. This results in an increased inversion, which takes place on the surfaces of the cells of the inner layers in the root, but which otherwise can be of the same kind. Such an explanation is in full accordance with the accepted picture of the free space and the conditions of a plasmolyzed tissue.

The increased inversion in hypotonic solutions of increasing concentration in the absence of calcium must in some way depend upon the decreasing tension of the tissue. Mannitol should be chemically inert, and it is difficult to see how it could directly increase the hydrolytic activity; a decrease by a competition of some kind would be more likely.

It is thus necessary to consider what happens when the turgor decreases, assuming that the reaction takes place in the free space in the cell walls. An increased inversion can be caused by (1) an increased size of the free space, or (2) an increased activity of the hydrolyzing system. The obtained results will be discussed from these points of view.

(1) *The size of the free space.* The free space must in the first hand consist of parts of the cell wall. According to Hylmö's (1958) deductions from the occurrence of the Erbe phenomenon in water uptake, the space available to a mass flow consists of the interfibrillary capillaries of diameters from about 20 mμ upwards. The space where a diffusion can take place may also comprise finer capillaries. It seems to be a reasonable assumption that the interstices change their form in an elastically tensed wall. Elastic tension of the wall means an increase in surface and decrease in thickness. The direction of the capillaries is predominantly in the tangential or oblique direction but not transversal. Thus the capillaries must increase in length and ought to decrease in width with increasing tension. According to the Erbe principle the pathways open to a solute movement must then decrease, or *the free space should decrease with increasing turgor tension*. This should decrease the surface inversion, which actually holds true.

With another and less stringent terminology this means that the "permeability" of the cell wall decreases. It may also be the same as the "conductivity" of Brouwer (1954). It is recognized that the cell wall is not so permeable as often has been assumed, particularly not to large molecules such as sucrose (Brauner 1956). Increasing permeability to solutes with decreasing turgor has been found by Bennet-Clark and Bexon (1943); but this has been connected by them with an increased cytoplasmic surface at concave plasmolysis. This does not hold true in the present instance, but the

superficial resemblance between the phenomena is striking. Rouschal and Strugger (1904) have suggested that the cytoplasm might regulate the mass-flow in the walls but proofs of this action are wanting. They show that plasmolysis rapidly changes the permeability of the cytoplasm, but it is undecided whether also wall properties are affected.

The sudden drop in inversion at incipient plasmolysis cannot be explained at present. It must depend upon some sudden change in the system, and the only one known to occur is the abrupt separation of wall and cytoplasm under partial rupture of the cytoplasm. What this means on a molecular basis is unknown: perhaps partial destruction of cytoplasmic systems, clogging of capillaries by cytoplasmic remnants etc.

The fact that the effect of hypertonic mannitol is inhibited completely by Ca could depend upon the strong regulation of hydration by Ca, influencing the porosity of the capillary system, or — with the classic terminology — a permeability-decreasing action of Ca.

If the above explanation of the turgor tension effect is right, it might also imply that decreasing turgor opens capillaries for passage with diameters only just allowing a flow of water under the given conditions. These finest capillaries ought in the first hand to be blocked by Ca^{+2} decreasing the permeability, or perhaps in some way mechanically at incipient plasmolysis.

(2) *The mechanism of sucrose hydrolysis.* In a balanced solution this reaction has a pH-optimum around 4.5. On surfaces with a high H-activity this should be shifted towards higher pH-values but the shape of the pH-curve remains unchanged (McLaren 1957). Mandels (1956) records a pH-optimum at 4.3 for surface-located invertase in *Verrucaria*. In the present instance removal of Ca shifts the optimum to about pH 7, or increases the activity 1000 times. There is as a matter of fact no pH-optimum at all, but the successive removal of Ca levels out the pH-curve. This seems to be an unusual kind of enzyme activity. It is worthwhile to consider the possibility that the surface hydrolysis is no enzyme reaction of the ordinary kind, but an acid hydrolysis caused by the high H-ion activity of the negatively charged cytoplasmic surfaces. The root surface also hydrolyzes maltose, although more slowly. Surfaces in this connexion, of course, also include the protein surfaces in the interior of the cell wall. It also seems as if it had not been possible as yet, in spite of numerous attempts, to isolate a saccharase in pure form (Sumner and Somers 1953). The results both with the H-ion dependance and the Ca-ion action can easily be explained in the way indicated, which was suggested earlier (Burström 1941). Removal of the strongly adsorbed Ca^{+2} causes an increased H-ion concentration in the outer ionic layer on the negatively charged surfaces. If the metal cations are completely removed, or practically so, which may be the case with EDTA, a wholly H^{+} -ion saturated surface

will result, which otherwise only could have been achieved in a pure acid. The surface activity then becomes independent of the pH of the external solution. This is apparently what takes place.

The decrease in rate of hydrolysis with decreasing turgor tension cannot depend upon an increase in H-ion activity, although the pressures in the cell wall are physiologically considerable. From known cell dimensions and osmotic data the tension in the epidermal cell walls can be estimated to about 10 and 20 atm. in the longitudinal and transversal directions respectively, according to the formula of Preston (1955), and in the root hairs to half these values. They are, however, far too small to affect others but the macromolecular or fibrillar structure, which has been discussed above.

(3) *The rate of the entrance of the solutes.* The principle of free space implies that the external solution moves freely in certain spaces, carrying the dissolved solutes outside the selective barriers. This may be the case in an intact plant with transpiration as the driving force of the movements. This is lacking in isolated pieces of tissue and the diffusion of the solutes remains as the only cause of the movements in the free space. As long as the sucrose hydrolysis takes place on the external surface solely, it is very sensitive to Hg^{+2} , but after plasmolysis the sensitivity decreases. This may be explained in the following way. Plasmolysis opens the pathways to cell layers in the interior, but the diffusion distances then also increase considerably. The great differences in diffusion potential for sucrose 10^{-3} and Hg 10^{-7} must then cause a large lead for sucrose over HgCl_2 , seemingly decreasing the sensitivity of the inversion to the inhibitor. A possibility which also must be considered is that HgCl_2 enters slowly, owing to binding of the strongly adsorbable cation to surface layers in the tissue. A decision between these two possibilities cannot be made at present, but there is no need for assuming a real change in the character of the reaction after plasmolysis.

Summary

The influence of additions of mannitol and some inhibitors on the surface inversion of sucrose has been studied on roots and coleoptiles of wheat. The following effects were observed on roots.

(1) In the absence of Ca hypotonic solutions of mannitol cause an increase in hydrolysis. (2) At incipient plasmolysis the hydrolysis rapidly decreases. (3) In hypertonic solutions the inversion increases. (4) $\text{Ca } 10^{-3} \text{ M}$ prevents the increase in hypotonic solutions and generally inhibits the hydrolysis. (5) The normal pH-curve has an optimum towards pH 4.5. Removal of Ca and addition of EDTA increases the hydrolysis particularly at pH 7, so that

the rate of hydrolysis becomes constant and high between pH 4.5 and 7. (6) HgCl_2 inhibits the hydrolysis. The inhibition is decreased in plasmolyzed tissue.

Coleoptile sections show a similar surface inversion of sucrose, with the same mode of reaction to Ca^{+2} and plasmolysis.

The results are explained on the assumption that plasmolysis opens up pathways to the interior of the root, which increases the hydrolysis. The other effects are tentatively related to changes in cell wall structure and charge of the surfaces where the inversion is supposed to take place. The bearings on the nature of the free space are discussed.

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Sodium and Potassium Distribution and Transport in the seaweed *Rhodymenia palmata* (L.) Grev.

By

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Introduction

The work described and discussed in this and a subsequent paper (MacRobbie and Dainty 1958) was undertaken to obtain quantitative information on the alkali ion (and chloride) content of and the fluxes between the various compartments of the plant cell and the external medium, under physiological conditions of experiment.

Progress in this field of plant physiology appears to have been slower than in the comparable field of ion transport in animal physiology. This is partly due to the difficulties of interpretation which arise from the morphological complication of the plant systems chosen for study *e.g.* excised roots. Also plant tissue is not usually in the same kind of steady state condition as animal tissue. For these reasons it has been possible less often in plant systems to express the results of ion transport studies in fully quantitative terms, as an ion flux in pmoles (10^{-12} moles) per sq.cm per second across a well-defined boundary separating two phases in which the electrochemical activities of the ions are known.

Marine algae appeared to have many advantages as experimental material for studies on the transport of ions with particular reference to the sodium-potassium distribution and *Rhodymenia palmata*, with its firm flat fronds, provided easily handled material. The rates of ion movements could be

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studied under physiological conditions by adding tracer quantities of the ion concerned to the normal sea-water medium; it was thus possible to study the material in its normal steady state. However this material has one serious disadvantage — a non-uniform cell population. The fronds of *Rhodymenia* are made up of small cells, about 10 μ diameter, near the surface and large cells, about 100 μ diameter in the interior; since the specific rate of exchange may be expected to depend on cell size this makes quantitative flux measurements difficult and uncertain.

Previous work on alkali metal ions in seaweeds has been done by R. Scott (1954), who studied caesium accumulation in *Rhodymenia*, and by G. T. Scott and H. R. Hayward (1953, a, b, c, 1954, 1955, 1957) who studied the sodium and potassium distributions and their metabolic connections in *Ulva lactuca*. R. Scott found a strong uptake of caesium in light, a process which was inhibited by azide, cyanide and CO_2 -deficiency. G. T. Scott and Hayward found in *Ulva* the usual high potassium, low sodium distribution, and studied the effects of light, iodoacetate, phenyl urethane, dinitro-*o*-cresol, arsenate, and ATP on the ion concentrations. From the results they concluded that the cellular sodium and potassium are regulated by different mechanisms, that the inward potassium pump is closely related to the glycolytic energy-yielding reactions and in particular to those involving phosphoglycerate, and that although the outward sodium pump seems to be coupled to the metabolism of pyruvate, it is not solely dependent on the generation of carbohydrates through photosynthesis, but has a more direct connection with light, perhaps by a redox mechanism of the type proposed by Conway (1953).

In the experiments to be described the rates of exchange of sodium and potassium between the seaweed and the surrounding sea water were measured under a variety of conditions by means of radioactive isotopes. In some respects the experiments are incomplete; the reasons for this will be discussed later. A brief account of some of the early experiments of this study has already been published (Dainty and MacRobbie 1955).

In this paper the terms *extracellular* and *intracellular* are used in the sense of *free* and *non-free space*. Thus the extracellular space would include the spaces between cells, the cell walls and any part of the cytoplasm open to free diffusion. Intracellular space would be the rest of the cytoplasm plus the vacuoles.

Methods

Plants were collected from North Berwick, Scotland, and were stored at 8°C for a limited time (up to a month) before use in aerated tanks of sea water from their natural habitat and illuminated for about 8 hours per day. Most of the experiments

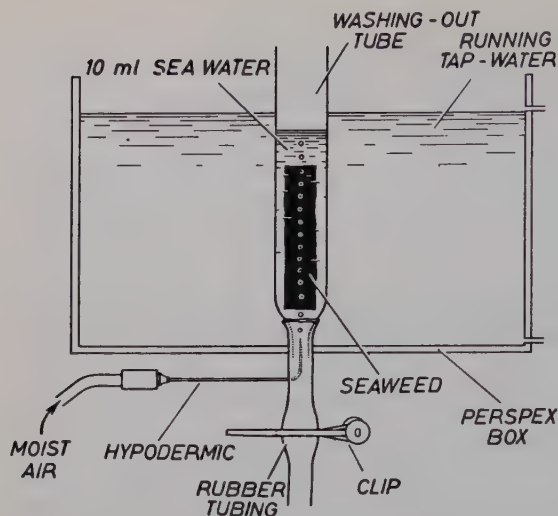


Figure 1. Diagram of the apparatus used in the washing-out experiments.

were done on rectangular strips cut from the fronds, 4 cm. by 1.2 cm. and weighing about 0.26 g. (wet weight); sometimes sets of six discs 1 cm. in diameter were used.

The isotopes used were ^{24}Na and ^{42}K , each of which was obtained from A.E.R.E., Harwell, as the spec.-pure carbonate. After conversion to the chloride by titration with N-HCl , the labelled salt was added to a suitable volume of sea water. This changed the concentration in the sea water by about 3 % for Na and about 12 % for K; an attempt was made to minimise the disturbance these changes created in the dynamic equilibrium of the cells by soaking the seaweed for 12–24 hours before the start of an experiment in an inactive modified sea water of the same chemical composition as the appropriate active sea water to be used. The radioactivity of the active sea water at the beginning of each experiment was about 20 $\mu\text{c}/\text{ml}$. for ^{24}Na -labelled sea water and about 0.1 $\mu\text{c}/\text{ml}$. for ^{42}K -labelled sea water. For ^{24}Na this represents an initial dose rate of about 20 rad/hour or a total dose of about 200 rad per experiment in the worst case; this might have decreased the fluxes somewhat (Barber, Neary and Russell 1957), but we had no indications from the experiments themselves that it did and we shall assume that there was no radiation effect on the fluxes.

The seaweed was placed in aerated, stirred, radioactive sea water for some time, usually overnight, until a suitable amount of the intracellular K or Na had exchanged with the radioactive isotope in the sea water. Usually both the amount of activity taken up in a given time and, by a 'washing-out' experiment, the subsequent loss of this labelled ion to an inactive medium were measured. In a washing-out experiment the strip of seaweed was transferred, after blotting lightly, from the active medium to 10 ml. of inactive modified sea water in a washing-out tube. This is shown in Figure 1 and consisted of a 10 cm. long pyrex tube, of internal diameter 1.5 cm. drawn down at one end to an external diameter of about 0.5 cm. where it was closed with a short length of rubber tubing and a spring clip. The 10 ml. of inactive sea water was stirred and oxygenated by moist air introduced by means

of a fine hypodermic needle thrust through the wall of the rubber tubing. At given times the sample was run out into a specimen tube by releasing the spring clip, and a fresh 10 ml. of inactive sea water poured into the tube. Thus each 10 ml. sample contained the ^{24}Na or ^{42}K lost from the seaweed in a specified time interval, and by measuring the radioactivity of each sample the rate of loss of ^{24}Na or ^{42}K was determined as a function of time. At the end of each experiment the amount of radioactivity left in the seaweed was found by wet-ashing it in 10 ml. of a 1:1 mixture of conc. H_2SO_4 and conc. HNO_3 and counting this sample.

The activities of the 10 ml. samples were determined by counting in M6 liquid counters using standard probe units, scalars and power units. In addition to the usual corrections for background, dead-time and radioactive decay, corrections were also applied for differences in self-absorption in liquids of different densities and for the amount of radioactivity left in the washing-out tube when a sample was run out. During the experiments the seaweed and the bathing solutions were maintained at a temperature of 8°C and in general two experiments, a 'light' experiment ('daylight' fluorescent lighting, 2,000 metre candles) and a 'dark' experiment (blackpainted covered tube), were run in parallel.

The amounts of Na and K in the seaweeds were determined by means of an EEL flame photometer. The material was first soaked in isotonic sucrose solution for 10 minutes to remove extracellular Na and K, then oven-dried for 24 hours at 110°C and finally dry-ashed in a platinum crucible in a muffle furnace for 24 hours at 450°C . The ash was dissolved in the minimum of dilute HNO_3 and this solution was suitably diluted for flame photometry. The intracellular chloride content was also measured on this solution by electrometric titration with AgNO_3 .

Results

Percentage of water in the seaweed

Comparison of the wet weight with the weight after oven-drying at 110°C for 24 hours gave the percentage of water in the seaweed. The water content was 80 ± 1 g. water/100 g. wet weight (10). [Such results will be quoted in the form: mean \pm S.E. of mean (number of results on which mean is based).]

Chemical determinations

These results represent the total Na, K and Cl in the seaweed after 10 minutes soaking in isotonic sucrose solution. It was assumed that ions remaining after this treatment were intracellular although the radioactive experiments later showed that these ions had a non-uniform exchange rate. The interpretation of this discrepancy will be discussed at a later stage.

The values found were: K, 415 ± 20 $\mu\text{moles/g. wet weight}$ (8); Na, 16.5 ± 1.5 $\mu\text{moles/g. wet weight}$ (8); Cl, 300 ± 20 $\mu\text{moles/g. wet weight}$ (6). The chloride value is likely to be an underestimate because of the possibility of loss during the ashing.

Radioactive measurements

Theory. — The equations governing the exchange of radioactive ions between a cell and the surrounding solution have been given by Ussing (1949) and others. A brief statement is necessary here before the results of the measurements are given.

If a cell containing no radioactive ions is placed in a large volume of solution in which the total external concentration of the ion species in question is C_0 and the external concentration of labelled ions is C_0^* , then the initial rate of entry of labelled ions into the cell is given by:

$$\frac{dc_i^*}{dt} = M_{in} \frac{A}{V} \frac{C_0^*}{C_0} \quad (1)$$

where A is the surface area, V is the volume, C_i^* is the internal concentration of labelled ions and M_{in} is the influx of the ion in moles/cm.²sec. After some time, when radioactive ions are crossing the cell boundary in both directions, the rate of change of internal activity is given by:

$$\frac{dc_i^*}{dt} = M_{in} \frac{A}{V} \frac{C_0^*}{C_0} - M_{out} \frac{A}{V} \frac{C_i^*}{C_i} \quad (2)$$

where C_i is the internal concentration and M_{out} is the efflux of the ion in moles/cm.²sec. In a washing-out experiment, in which the radioactive ion is not allowed to accumulate in the external medium, C_0^* may be taken as zero at all times and the solution of equation (2) is thus:

$$C_i^* = C_{i0}^* \exp \left(\frac{-M_{out} A}{C_i V} \cdot t \right) \quad (3)$$

where C_{i0}^* is the initial concentration of labelled ion in the cell. Thus if $\ln C_i^*$ is plotted against time the graph will be a straight line of intercept $\ln C_{i0}^*$ and slope $M_{out} A/VC_i$. The slope, which we shall call k , allows calculation of the efflux provided the internal ion concentration and the surface/volume ratio of the cell are known. The influx may be calculated from the uptake over a period short enough for the internal radioactive ion concentration to be considered negligible, by means of equation (1).

With a tissue such as *Rhodymenia*, the exchange of ions is, in principle, more complicated than the above equations suggest. The exchange is at least a two-component process: a rapid exchange by diffusion from the extracellular spaces and a much slower exchange across the protoplasmic membranes. Mathematical solutions of this two-component process have been given, for special cases, by Harris and Burn (1949), and Dainty and MacRobbie (unpublished). These show that, provided the rates of the two processes are sufficiently different, their separate contributions may be calculated and a graph of the amount of radioactivity against time (in a washing-out experiment) reduces to the sum of an exponential, whose time constant is governed by the

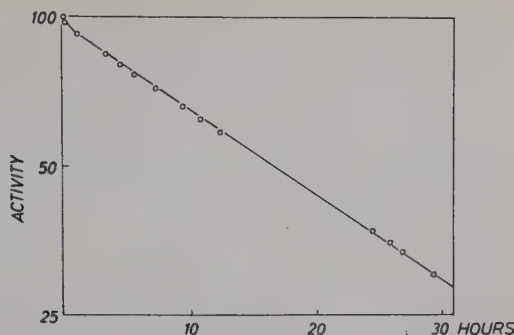


Figure 2. *Exchange of radioactive potassium; activity left in the seaweed plotted logarithmically against time.*

rate of exchange across the protoplasmic membrane, and a diffusion curve, governed by the rate of diffusion from the extracellular space. The conditions for adequate separation of these two components are amply fulfilled in *Rhodomenia* and one would therefore expect the semi-log plot of a washing-out experiment to reduce to the sum of two straight lines (since a diffusion curve is exponential after a brief initial period), whose slopes are related to the rates of the diffusion and exchange processes and whose intercepts are related to the amounts of extra- and intracellular ion.

These two fractions were in fact found, but the intracellular component appeared to have a non-uniform rate of exchange and this was more marked for Na than for K.

The interpretation of the experimental curves for Na and K will be considered separately.

Potassium. — A typical 'washing-out' curve of the amount of radioactive potassium left in the seaweed plotted logarithmically against time is shown in Figure 2. This strip had taken up radioactive potassium for 40 hr. and it can be seen that after this time over 90 % of the radioactivity was in the slowly exchanging fraction and therefore probably intracellular.

Intracellular potassium. The intercept of the 'slow' straight line on the axis $t=0$ represents — so we shall argue — the initial intracellular radioactivity; the percentage of exchange during the time of uptake may be calculated from the rate of exchange of this slow fraction and hence the amount of exchangeable potassium in the cells may be obtained. The uptake times were such that 40—95 % of the total intracellular potassium was labelled at the start of a washing-out experiment. The value found for intracellular potassium was 315 ± 15 $\mu\text{moles K/g wet weight}$ (8).

Extracellular space. The contribution of the intracellular fraction to the total radioactivity in the initial rapid exchange is estimated from the slow component and is then subtracted from the total; the remainder is replotted,

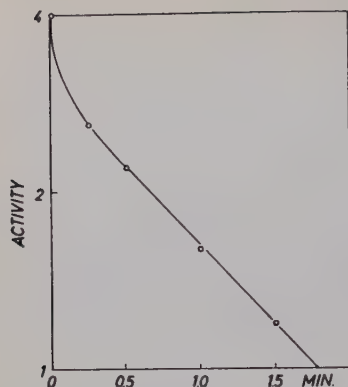


Figure 3. Exchange of extracellular potassium; extracellular activity plotted logarithmically against time.

again logarithmically, against time to find the amount of extracellular potassium and its rate of exchange. A typical result is shown in Figure 3; this fits a diffusion curve in which the intercept of the straight line part of the curve should have the value $8/\pi^2$ times the amount of K in the extracellular space, and its slope should have the value $\pi^2 D'/l^2$, where D' is the apparent diffusion coefficient in the extracellular space and l is the thickness of the seaweed strip. It was assumed that the concentrations in the extracellular space were the same as those in the external sea water and hence the volume of the extracellular space was calculated. This volume was found to be 0.237 ± 0.008 ml./g. wet weight (14). From the slope D' was found to be $(3.6 \pm 0.4) \cdot 10^{-6}$ cm²/sec. The hindrance to free diffusion imposed by the presence of cells in the tissue is reflected in the difference between this value and the value 16×10^{-6} cm²/sec. — the probable diffusion coefficient of K in sea water. It should be stated here that the values for the extracellular volume calculated from the sodium and potassium experiments were in agreement.

Intracellular concentration. From the amount of water in the seaweed and the volume of the extracellular space, the amount of intracellular water may be calculated. Thus in 1 g. of tissue (wet weight) the weight of total water is 0.80 ± 0.01 g., of extracellular water 0.234 ± 0.008 g., and hence the weight of intracellular water is 0.566 ± 0.015 g. Using this value and the value of 315 ± 15 μ moles/g. wet weight for the intracellular K, we find the average intracellular K concentration in the cell water to be 560 ± 40 μ moles K/g. cell water.

Rate constants for K exchange across the protoplasmic 'membrane'. — Although the rate constant k ($=M_{out}A/VC_i$) for the exchange across the protoplasmic membrane may be estimated from the slope of the slow part of the washing-out curve, a more accurate value, given our experimental method,

Table 1. *Rate constants (10^2k hr $^{-1}$) for potassium exchange across the cell 'membrane'.*

Conditions	Over $t = 0$ to 1 hr		Over $t = 2$ to 12–40 hr	
	Range	Mean	Range	Mean
Light (2000 metrecandles)	5.0 — 12.0	7.0 ± 1.2 (6)	3.8 — 5.6	4.8 ± 0.3 (5)
Dark	0.66 — 2.16	1.3 ± 0.2 (7)	0.38 — 0.68	0.48 ± 0.06 (5)

may be found by calculating the specific rate of exchange for each time interval. This is $\frac{1}{c_i^*} \frac{dc_i^*}{dt}$ or $\frac{\text{counts/min lost per unit time}}{\text{counts/min present in seaweed}}$. By this means any apparent variation of k with time is clearly seen and it is possible to compare closely the behaviour of the intracellular fraction with that of a simple compartment whose ions exchange at a single uniform rate. In fact some deviation from this simple system was found and the specific rate of exchange of the intracellular potassium was higher during the first hour's washing-out than the steady value assumed in the later part of the experiment. The values found for k are summarised in Table 1; the values over the first hour were obtained from the slope of the curve during that period, but the final steady rate constant for each strip of seaweed was calculated as the mean of the values during a number of time intervals, usually from 5 to 10 points over a period of 5–12 hours at least. For individual strips of seaweed the standard error of the mean calculated from the values in these time intervals was never more than 12 %, and was usually 1–5 %. The range of k values for different strips is given, together with their mean. Rates of exchange in both light and dark are quoted since the effect of illumination is very marked.

Since the rate constants for different strips of seaweed show considerable spread the most reliable demonstration of the effects of illumination, or of any other specific factor, is the change in rate of exchange of a given piece of seaweed on the appropriate change in conditions. That the efflux is much faster in light than in dark is clearly seen both from the values of Table 1 and from the graph of Figure 4. The rate constants for this latter experiment are given in Table 2, together with those for the parallel experiment in which the light-dark periods were interchanged.

It was noticed that when the change to light was made after a prolonged period of darkness k rose gradually to a maximum before falling slightly to a steady value, whereas after only a short period of darkness the full 'light' value of k was attained immediately on re-illumination. Similar differences were seen in the effects of the change from light to dark; after a prolonged period of light the rate of exchange did not fall immediately to the typical 'dark' value but remained rather higher for at least four hours, whereas with

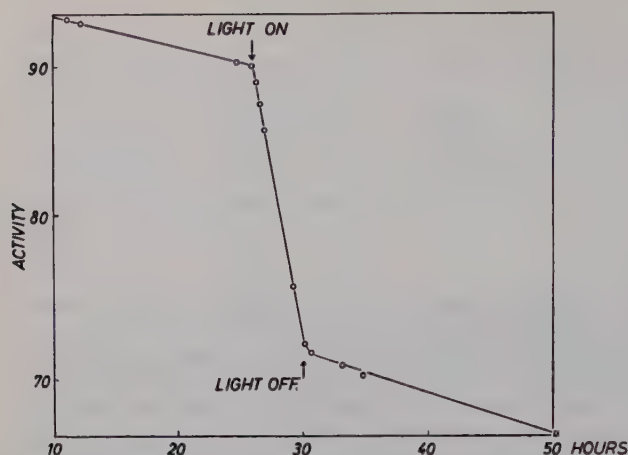


Figure 4. Exchange of intra-cellular potassium in light and dark.

a dark-adapted seaweed the normal 'dark' value was reached within two hours of the return to dark after a short period in the light.

Potassium fluxes. The efflux of potassium from the cells may be calculated from the results of the washing-out experiments from the formula:

$$M_{\text{out}} = k \frac{V}{A} C_i \quad (4)$$

There are serious difficulties in applying this formula to the results described above, all arising from the uncertainties inherent in measurements on a non-uniform cell population. The volume/surface ratio of a spherical cell has the value $d/6$, where d is the cell diameter, and hence varies directly with the cell size. Both the other quantities, k and C_i , may be expected to vary with cell size. The most probable explanation of the variation of k over the course of a washing-out experiment is that the small cells of the population exchange more rapidly than the large ones and therefore contribute a disproportionate amount to the early part of the exchange; the amount of potassium in this 'small cell' fraction is not large — only a few percent of the total slow fraction.

Table 2. Rate constants ($10^2 k \text{ hr}^{-1}$) during light-dark periods for two strips of seaweed, A and B.

Time	A		B	
	Light	Dark	Light	Dark
From 2 to 26 hr. ...	4.9 ± 0.3 (7)	0.38 ± 0.02 (9)	5.58 ± 0.05 (13)	0.98 ± 0.05 (5)
From 26 to 30 hr. ...				
From 30 to 40 hr. ...		0.44 ± 0.05 (4)	5.6 ± 0.1 (6)	

An estimate of the efflux from the large cells may be obtained from the k value for the period 2—12 hr., V/A for the large cells, the total exchangeable potassium in the slow fraction and an estimate from histological sections of the volume of tissue occupied by the large cells. This gives an efflux of 18 pmoles $K/cm.^2sec.$ in light and 1.8 pmoles $K/cm.^2sec.$ in the dark. These estimates are probably reliable to within a factor three, although the light/dark ratio is much more reliable.

It is not possible to calculate an influx figure comparable with the efflux given above. The influx into the cells must be calculated from the intracellular uptake of labelled ion in a time, short compared with the halftime for exchange across the protoplasmic membrane, during which the internal concentration of labelled ions remains small enough for their outward movement to be neglected. The rate of entry of labelled ion is then given by equation (1) and the influx takes the form:

$$M_{in} = \frac{1}{A} \frac{C_o}{C_o^*} \frac{d}{dt} (VC_i^*) = \frac{C_o}{C_o^*} \frac{Q^*}{At} \quad (5)$$

where Q^* is the amount of labelled ion taken up in time t . However at short uptake times the effects of the small, more rapidly exchanging cells become more important, and the compartment involved in the exchange is ill-defined. Over periods of 1—2 hr., uptakes of 8—11 $\mu moles K/g.$ wet weight/hr. in light and 1.8—4.0 $\mu moles K/g.$ wet weight/hr. in the dark were found. The figures for the outward movement of potassium most nearly comparable with these are obtained by multiplying the mean rate constant over the first hour of a washing-out experiment by the amount of intracellular potassium; this gives outward movements of 19 $\mu moles K/g.$ wet weight/hr. in light and 4 $\mu moles K/g.$ wet weight/hr. in the dark. Thus the movements inwards and outwards are of the same order of magnitude, but since the method of calculating the outward movement is open to a number of objections if the two figures are to be compared, it is not justifiable to draw any conclusions from the differences between them.

Effects of dinitrophenol. Two experiments involving six strips of seaweed were done on the effects of DNP at a concentration of $4 \times 10^{-4} M$. The efflux experiments were done by allowing the seaweed to take up K under normal conditions and then following the rate of loss of activity during three successive 4 hr. periods in sea water, sea water + DNP, sea water. In light the efflux was reduced in DNP by a factor of 0.77 ± 0.02 . In the only experiment done in the dark there was a reduction of the efflux by a factor of 0.83. The influx in DNP-sea water was also compared with that in normal sea water. In light the influx in DNP was reduced by a factor of 0.81; the effect on the influx in the dark was not measured.

Table 3. *Effects of added glutamate on potassium fluxes in the dark.*

Glutamate concentration mM	Ratio: <i>efflux in sea water + glutamate</i> <i>efflux in sea water</i>	Ratio: <i>influx in sea water + glutamate</i> <i>influx in sea water</i>
10	2.0 ± 0.2	not measured
25	2.3 ± 0.2	2.4
50	3.4 ± 0.1	not measured

Effects of glutamate. Solid sodium glutamate was added to modified sea water to give glutamate concentrations of 10, 25 or 50 mM. The pH of all such solutions was adjusted to the normal sea water pH. The experiments were always done on two strips of seaweed cut from the same frond and 'normal' and 'glutamate' treatments were alternated. The results showed that the addition of glutamate had no effect on the efflux of potassium in the light, but it had marked effects on the efflux (and influx) in the dark. The results are collected in Table 3.

Sodium. — The treatment of the experimental results for sodium is similar to that for potassium; the washing-out curves are basically similar but differ in the relative amounts of the various fractions. As for potassium the extracellular fraction was found by subtraction of the estimated intracellular radioactivity from the total activity, but for sodium about 90 % of the total Na was extracellular. (See Figures 5, 6, 7.)

Extracellular space. The loss of sodium from the extracellular space fitted a diffusion curve and the amount of sodium and the apparent diffusion coefficient of sodium in this fraction were found from the straight line semi-log. plot of the latter part of this diffusion curve (Figure 5). It was assumed that the sodium concentration in the extracellular space was the same as that in the external sea water and the volume of the space was calculated. Some seasonal variation in the volume of the extracellular space and the apparent diffusion coefficient was found; the extracellular volume of 'summer' seaweed was 0.20 ± 0.01 ml./g. wet weight (7) and of 'autumn' seaweed 0.237 ± 0.008 ml./g. wet weight (10); the corresponding apparent diffusion coefficients were $(4.7 \pm 0.3) \times 10^{-6}$ cm.²/sec. and $(3.3 \pm 0.1) \times 10^{-6}$ cm.²/sec. The second figure for the extracellular volume agrees exactly with the figure quoted already for potassium experiments on the same type of seaweed. The exactness of the agreement is of course fortuitous, but it does support the assumptions on which the calculation of extracellular volume is based. The apparent diffusion coefficients for Na in the extracellular space are less than the value in sea water (10×10^{-6} cm.²/sec.), thus again illustrating the effect of the presence of cells as a hindrance to diffusion.

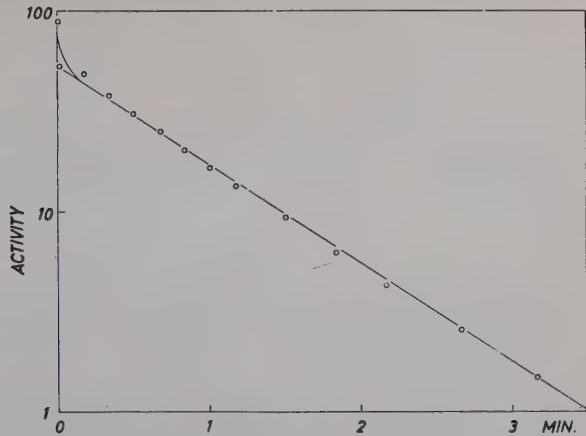


Figure 5. *Exchange of extracellular sodium.*

Intracellular sodium. The total amount of intracellular activity may be found from the intercept at $t=0$ of the slow fraction in the semi-log. plot of a washing-out experiment (see Figure 7). The mean value for the total intracellular sodium, from 9 experiments, was 14 ± 1 μ moles Na/g. wet weight (9).

Since the non-uniformity of the exchange rate was so marked, a further analysis of the washing-out curve allowed a better characterisation of the intracellular sodium and its exchange rate. As in the potassium experiments the rate constant k fell to a final steady value after about 2 hours of washing-out, but the amount of sodium in this final slow exchange was appreciably less than the total intracellular sodium. It was possible to find a second intracellular fraction with a more rapid rate of exchange by subtracting the con-

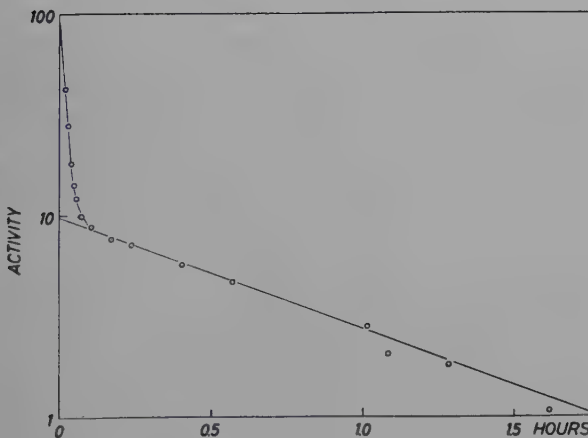


Figure 6. *Exchange of sodium in the fast intracellular fraction II.*

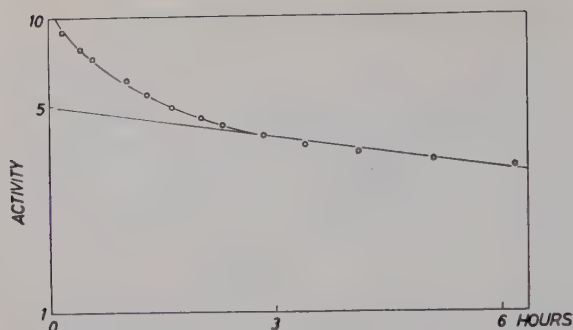


Figure 7. *Exchange of intracellular sodium; the straight line represents the exchange with the slow intracellular fraction I.*

tribution of the slowest fraction from the total intracellular activity and plotting the remainder logarithmically against time. An example of the contributions of the three fractions, extracellular Na and the two intracellular Na fractions, is shown in Figures 5, 6, 7. The mean value of the amount of Na in the slow intracellular fraction was 9.0 ± 1.3 μ moles Na/g. wet weight (8); and in the fast intracellular fraction — 5.0 ± 0.5 μ moles Na/g. wet weight (8). (After the usual uptake times only the slowest fraction was incompletely labelled and allowance for this was made in the calculation of the total Na from the amounts of labelled ion.)

The mean concentration of sodium in cell water was found from the total intracellular sodium and the total intracellular water to be 25 ± 3 μ moles/g. cell water. The intra- and extracellular Na and K concentrations, from radio-activity measurements, are collected in Table 4.

Rate constants for sodium exchange across the cell membrane. The efflux rate constants for Na exchange in light and in dark are summarised in Table 5 for both fraction I, the slowly exchanging cells, and fraction II, the more rapidly exchanging cells.

The ratios of the mean value of k in light to the mean value of k in the dark are 1.5 ± 0.3 in the slow cells and 1.8 ± 0.2 in the fast cells. Experiments in which the effect of light and dark was especially studied gave similar values; in three experiments six values of the ratio were obtained whose mean was 1.5 ± 0.1 (6); there was no systematic difference between the slow

Table 4. *Ion concentrations in μ moles/g. water.*

Ion	Mean intracellular	Extracellular
Na	25 ± 3	467
K	560 ± 40	11.0

Table 5. *Efflux rate constants (10^2k hr^{-1}) for sodium exchange across the protoplasmic 'membrane'.*

Conditions	I		II	
	Range	Mean	Range	Mean
Light	24 — 39	30 ± 4 (4)	76 — 180	130 ± 10 (8)
Dark	15 — 26	19 ± 2 (4)	59 — 99	74 ± 6 (8)

and the fast cells. These results may be combined in the statement that the sodium efflux is greater in light than in the dark by a factor in the range 1.3 to 1.8, with a mean value of 1.5 ± 0.1 (8).

Sodium fluxes. Since the slow and fast intracellular fractions were separable, the total inward and outward movements in the cells of 1 g. of tissue may be found with reasonable accuracy. The outward movement (in $\mu\text{moles/g. wet weight/hr.}$) from the cells, M'_{out} , is given by:

$$M'_{\text{out}} = M'_{\text{Iout}} + M'_{\text{IIout}} = k_1 Q_1 + k_2 Q_2 \quad (6)$$

where k_1 , k_2 and Q_1 , Q_2 are the rate constants and the amounts of Na in the respective intracellular fractions. The values of M'_{out} and its component parts, in light and in dark, are given in Table 6. Also given in the same table are the inward sodium movements, M'_{in} , in light and in dark, calculated from the amount of labelled Na taken up during a short time. Periods of 10—30 min. were used, but since in light there was appreciable exchange in this period, the equation

$$M'_{\text{in}} = Q^* \frac{kt}{1 - e^{-kt}} \quad (7)$$

was used to calculate the inward movement. (Q^* is the uptake per unit time and k is the rate constant for washing-out determined for the same strip over time t , the uptake time.) From these figures the assumption that the tissue is in a steady state appears to be justified.

Table 6. *Sodium movements, M' , in $\mu\text{moles Na/g. wet weight per hour}$, in the slow cells (I), the fast cells (II), and the whole tissue.*

Movements	Light	Dark
$M'_{\text{I out}}$	2.4 ± 0.4	1.6 ± 0.3
$M'_{\text{II out}}$	7.0 ± 1.1	4.9 ± 1.0
$M'_{\text{out}} = M'_{\text{I out}} + M'_{\text{II out}}$	9.4 ± 1.2	6.5 ± 1.0
M'_{in}	10.1 ± 0.5 (3)	6.8 ± 0.5 (6)

Table 7. *Approximate efflux from large cells in pmoles/cm.² sec.*

Conditions	Na	K
Light	1.8	18
Dark	1.2	1.8

If the slow fraction is attributed to the large cells of the population, as seems reasonable, approximate values for the Na efflux may be calculated from these figures as was done for the K efflux. These are given in Table 7, together with the potassium figures already given; they are both uncertain by about a factor three.

The effects of DNP and glutamate on the sodium movements in *Rhododymenia* have not been studied.

Discussion

While this work allows some conclusions to be drawn on the sodium-potassium distribution and its maintenance in *Rhododymenia*, accurate flux figures cannot be quoted, nor can any really reliable assessment be made of the contributions of different cellular phases to the ion distribution and transport. It is for these reasons that in some respects the work was left incomplete and many interesting and important problems were not followed up.

The amounts and rates of the very fast initial exchange are consistent with a loss by diffusion from the extracellular spaces. Both sodium and potassium diffusion coefficients in the extracellular space are less than the probable values in sea water, as is expected in a tissue where the presence of relatively impermeable cells hinders free diffusion both by reducing the effective area for diffusion and by lengthening the diffusion path. The ratio of the potassium to the sodium diffusion coefficient is also reduced, but we cannot suggest why. The volume of the extracellular space was calculated on the assumption that it is filled with sea water and the agreement between the sodium-space and the potassium-space supports this assumption. The extracellular space should have been determined for molecules other than monovalent cations, but this was not done. The presence of a very high immobile anion concentration in the free space would raise the mobile cation concentration and lead to an overestimate of the extracellular volume by this method, but it is thought that any serious error arising from neglect of this possibility is unlikely. There is known to be a relatively large extracellular space in the tissue in which the ion concentrations must have the external values, and the

immobile anion concentrations in any additional Donnan phase would need to be exceedingly high compared with an already high external concentration to change seriously the total amount of ion in the combination of the two phases.

The interpretation of the intracellular fractions and their heterogeneous rates of exchange presents difficulties. As has been stressed already it is not to be expected that the exchange in a non-uniform cell population should be controlled by a single rate constant. In addition each cell probably contains at least two phases — 'protoplasmic non-free space' and vacuole (MacRobbie and Dainty 1958) — in which the ions have relatively slow rates of exchange. We think it reasonable, however, to assume that the final steady value of the rate constant in a washing-out experiment represents loss from the vacuoles of the large cells of the population, those in the centre of the flat thallus, and that the initial faster rate represents loss from the smaller cells (and, possibly, from the protoplasmic non-free space of all the cells). This implies that the vacuoles of the large cells would contain about 310 μ moles K and 9 μ moles Na, per g. tissue wet weight. (Here we are taking the intracellular potassium determined from the radioactive measurements; this is exchangeable potassium and presumably exists as free potassium ions in solution in the vacuole. The electrochemical potential will be determined by this free potassium. The difference between the chemical and radioactive measurements suggests that about 100 μ moles K/g. tissue wet weight is bound in an unexchangeable form.) From tissue sections it was estimated that about $\frac{2}{3}$ of the intracellular water is in the large cells — say 0.4 ml./g. tissue wet weight — and hence the intracellular K and Na concentrations will be about 800 mM and 25 mM respectively. The principal balancing anion is presumably chloride for chemical determinations show that the intracellular chloride is about 75 per cent or more, on a molar basis, of the intracellular potassium.

After earlier failures during the course of this work it has recently been found possible to insert KCl microelectrodes into the large cells and thus measure the potential difference between vacuole and external sea water (Johnston and Williams, in this laboratory, private communication). Measurements on twelve cells gave a fairly constant value of -65 mV (vacuole negative) and this enables us to discuss which ions are actively transported. For purely passive exchange between two phases the following relation between ion concentration (strictly activity) and equilibrium potential difference must hold:

$$E = \frac{RT}{zF} \ln \frac{C_o}{C_i} = \frac{58}{z} \log_{10} \frac{C_o}{C_i} \text{ mV} \quad (8)$$

where E is the potential difference between inside (i) and outside (o) and z is the charge on the ion in electron charges. The equilibrium potential dif-

ferences for K, Na and Cl calculated from this equation are approximately -110 mV, $+75$ mV and zero respectively. Comparison with the observed potential difference of -65 mV shows that the electrochemical potentials of K and Cl are higher inside than outside but that of Na is much lower inside than outside. If we assume that the cell membranes are permeable to Cl these figures suggest that K and Cl are actively transported *into* the cell (vacuole) and Na is actively transported *out*. As with animal cells (Harris 1956) Na, for which both concentration and potential gradients are directed inwards, is farthest from electrochemical equilibrium but, unlike most animal cells, Cl must be 'pumped' into these plant cells. This latter point is discussed in some detail in a later paper (MacRobbie and Dainty 1958), where more quantitative information is available on another cell. K is fairly close to electrochemical equilibrium and may be closer than the figures suggest, for a fairly large, tough, microelectrode, of tip diameter $2-3\ \mu$, had to be used to penetrate the cell walls; this may damage and somewhat depolarise the cell and this would result in a lower potential reading, though there was little sign of this, a potential of -65 mV being maintained for some hours.

The K efflux, in light, from the large cells is of the same order of magnitude as in squid nerve and frog muscle (Harris 1956); the Na efflux, however, is about ten times smaller. If, in the light, the influxes are equal to the corresponding effluxes and if both the K and Na influxes are purely passive (though there is good reason to doubt this), then the cell 'membrane' is about 400 times more permeable to potassium than to sodium. This is a much higher permeability ratio than the usual 20—100 figure in other cells (Conway 1957).

The apparent energy requirement for extruding Na from the cell, assuming that all the Na efflux is due to active transport, is about 5×10^{-7} cal/hour/large cell or, if $1/10$ of the cell mass is protoplasm, 0.1 cal/hour/g. protoplasm; (see Keynes and Maisel 1953, for method of calculating energy requirement). This value is quite high and to it must be added an unknown amount for pumping chloride, and perhaps potassium, into the cell; it therefore suggests that part of the fluxes may be due to exchange diffusion (Ussing 1947) which would reduce the energy requirement.

The effect of light is to increase the rates of exchange of both K and Na but it is most pronounced on the rate of K exchange in the large cells, where the efflux in light is about eight times the 'dark' value and the influx is also greater. It was not possible to decide whether the two fluxes changed in exactly the same proportion; probably (see Scott and Hayward 1955) the efflux is greater than the influx in the dark, giving a net loss of potassium. Though there may be some active transport of potassium inwards, the fluxes are probably mostly passive or due to exchange diffusion. If the fluxes are purely passive, *i.e.* K ions are diffusing across the membrane as ions, then

the light must directly affect the permeability of the cell membrane, possibly by widening water-filled pores, though not enough to affect the sodium fluxes appreciably. It is more attractive to attribute the K fluxes to exchange diffusion and the effect of light to a great increase in the supply of K carriers. The time course of the light-dark effect is compatible with the building up of carriers during photosynthesis in the light and their removal by respiration in the dark.

In view of the fact that sodium must certainly be actively transported out of the cell the light-dark effect on the sodium fluxes, and particularly on the efflux, is surprisingly small. This suggests that Na transport is linked much more closely with respiration than with photosynthesis. It is possible to speculate about these links but this would be unrewarding until the necessary further experiments on the effect of metabolic inhibitors have been carried out. Their effects on Na transport have not been studied and the few experiments on the effect of DNP on the K fluxes are not very informative; the effect was small.

The effects of glutamate on the K fluxes in the dark are most readily explained by supposing that glutamate stimulates the formation of carrier molecules. This need not imply that glutamate itself or a close derivative is the carrier; glutamate has extensive metabolic connections and may be transformed into a variety of substances occupying key positions in the cell metabolic schemes. The effect of other amino-acids and of substances related to the other aspects of glutamate metabolism might suggest in what capacity glutamate is acting.

The discussion so far has been concerned with the slowest exchanging fraction containing the bulk of the intracellular K and 60 % of the intracellular Na, which we have reasonably associated with the vacuoles of the large cells. The faster exchanging intracellular fraction is probably of a more heterogeneous origin and we consider that speculation about its characteristic properties — Na/K ratio, effect of light, etc. — is unwarranted in view of the uncertainty of its morphological position.

The work discussed in this paper goes some way towards fulfilling the aim with which it was undertaken, which was to obtain quantitative information on the ion content of the various compartments of the tissue and the ion fluxes between them and the external medium. But as long as it is necessary to allow for the effects of a non-uniform cell population in interpreting the results it is not possible to separate the behaviour of different subcellular compartments; the ionic relations within the cell involving the protoplasm, protoplasmic inclusions and vacuole, though of fundamental importance cannot be distinguished from the effects of variations in cell size. It was there-

fore thought that work on a tissue morphologically less complicated would be of greater value and attention was transferred to the giant Characean cells; this work is reported in subsequent papers.

Summary

The sodium and potassium distribution and transport in *Rhodymenia palmata* have been studied using ^{24}Na and ^{42}K . Chemical measurements showed that the seaweed contained 0.80 ± 0.01 g. water, 415 ± 20 μmoles intracellular potassium, 16.5 ± 1.5 μmoles intracellular sodium and 300 ± 20 μmoles intracellular chloride, all per g. tissue wet weight. Radioactive determinations of intracellular exchangeable ion contents gave 315 ± 15 μmoles K and 14 ± 1 μmoles Na per g. tissue wet weight. The tissue contained an extracellular space, filled with sea water and open to free diffusion, of amount 0.24 ± 0.01 ml./g. tissue wet weight.

Kinetic studies of the exchange of K and Na between the tissue and the external sea water indicated that there was more than one intracellular compartment in the tissue. However most of the potassium and 60 % of the sodium showed a uniform slow exchange rate and this compartment was assigned to the large central cells of the fronds which contain about $\frac{2}{3}$ of the intracellular water. There was a marked light-dark effect on the potassium fluxes, but only a small effect on the sodium fluxes. Estimates of these fluxes are given and the effects of DNP and glutamate on the potassium fluxes were studied.

The results are discussed in terms of current ion transport theories and the difficulties of working with non-uniform cell populations are stressed.

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On the Relation between Turgor Pressure and Tissue Rigidity. I

Experiments on Resonance Frequency and Tissue Rigidity

By

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I. Introduction

Virgin (1955) has introduced a new method for the determination of the turgor of plant tissue by measuring their resonance frequencies. The resonance frequency of a plant tissue changes with the turgor of the tissue. In the present paper it will be shown that the turgor pressure in the parenchyma of potato tuber can be measured by this method and that this turgor pressure affects the mechanical properties of the tissue. First an account is given on the determination of the plastic and elastic component in the potato tuber parenchyma and the determination of the turgor pressure. Then follows the determination of resonance frequencies and it is shown that there is a relation between these frequencies and the turgor pressure. For the sake of comparison also direct determinations of the tissue rigidity of the tissue at different turgor pressure are given, using a conventional method.

Abbreviations:

M moles per litre.
v resonance frequency.
 v_c corrected resonance frequency.
p/s periods per second.

d thickness.
l length.
 l_0 length, when $O_0 = \text{zero}$.
 Δl change in length.

p	turgor pressure.	q	cross section.
O _e	osmotic value of the surrounding solution.	F	stretching force.
O _c	osmotic value of the cell contents.	I	moment of inertia.
E	Yqung's modulus (modulus of elasticity).	y	amplitude at x.
R	constant due to water friction.	x	distance from the fixed end.
ρ	density of the material.	m	mass of the material.
		t	time.
		K, k, κ , a, b and s constants.	

II. Relation between Turgor Pressure and Resonance Frequency

Material and method

Pieces of parenchyma from healthy potato tubers, stored at about $+5^{\circ}\text{C}$, have been used as material for this study. The pieces were cut out from the layers inside the main cambium. The parenchyma of the potato tuber consists of more or less isodiametrical cells, with very small intercellular spaces. The average diameter of the cells was found to be about 0.1 mm. at ordinary turgor pressure. The density of the tissue was very close to one.

At the determination of the resonance frequencies a small spiral of piano-wire, 0.25 mm. in diameter, was applied at one end of the rodshaped pieces of parenchyma. The spiral weighed about 2 mg. and had been coated with plastic to prevent rusting. For this plastic-coating a solution of "Bonoplex"-plast in trichlorethylene was used, in which the spiral was dipped and then allowed to dry for 24 hours so that all solvent had evaporated. With the aid of a barely melted mixture of 1 part beeswax and 4 parts cacao butter, which was allowed to solidify, the other end of the parenchyma-rod was fastened into a glass tube. That part of the parenchyma, which was free from the tube, was, when not otherwise stated, about 8 mm. long as measured from the opening of the glass tube to the middle of the spiral. The glass tube was fastened in a holder, which was placed on the edges of a Beckman cell in such a way, that the tube with the plant material was inside the cell. The cell, which held about 4 ml., was then filled with a water solution which contained 10^{-5} M CaCl_2 and 10^{-5} M K_2SO_4 and sometimes also mannitol. Outside the Beckman cell and in front of the iron spiral an electromagnet with an iron-core was placed, which was connected with an oscillator, giving an alternating sine-formed current. The current could be regulated as to intensity and frequency. The free end of the plant parenchyma was observed through a horizontal microscope of 40–50 times magnification, and the frequency giving the maximal amplitude was determined. Because of the varying damping effect of the surrounding solution it may sometimes be difficult to determine the resonance frequency, but usually this can be done with great accuracy.

Since the iron core of the magnet showed no permanent magnetization it should be observed, that the oscillation frequency of the rod is twice the frequency of the alternating current supplied to the magnet. This is due to the fact that the absolute magnitude of the magnetic field generated by the magnet varies with double the frequency of the alternating current.

The same material can, however, show several different more or less distinct resonance frequencies. These are of three principally different kinds. 1. the

fundamental resonance frequency, 2. higher harmonics and 3. false resonance frequencies. The higher harmonics, which are not harmonious in the case of a vibrating rod, are caused by the material vibrating with two or more nodes. The second harmonic is 6.27 times the fundamental frequency (Bergman and Schaefer 1954). The false frequencies are formed because of the fact that the frequency of the disturbing force has only to be a multiple of the fundamental resonance frequency to bring about resonance. These frequencies are harmonious and the ratios between the next and most noticeable false resonance frequencies and the fundamental resonance frequency are $\frac{2}{5} : \frac{1}{2} : \frac{2}{3} : 1 : 2 : 3$. The amplitude of these other

reconance frequencies is always lower than that of the fundamental resonance frequency. Therefore, to find the fundamental resonance frequency for a certain material, one has first to determine the different resonance frequencies of that material and only by studying the mutual relation between these it is possible to decide which one of them is the fundamental one. The intensity of the alternating current must be kept so low, that the amplitude of the vibrations becomes as small as possible but nevertheless allows great accuracy when determining the resonance frequency. It is of importance that the bending vibrations do not exceed the limit of elasticity or otherwise the value received for the resonance frequency will be too low. As experiments have shown, however, that this source of error will become actual only for very large amplitudes, it can be left out of consideration.

The coil of an electromagnet, through which an alternating current flows, changes its impedance with the frequency of the alternating current, and therefore the electric circuit of the magnet may itself show resonance peaks. The intensity of the magnetic field is then increased, which may cause that extra resonance frequencies due to the magnet only are observed. This source of error can cause some difficulties of measuring the resonance frequencies if unsuitable magnets are chosen. The position of these extra resonance frequencies due to the magnet only can, however, easily be found by determining the frequency of the alternating current from the oscillator, which gives a maximum of the strength of the current.

The measurements have been made within a frequency range of 60—260 p/s. The temperature was kept at 20—22°C, unless otherwise stated.

Results

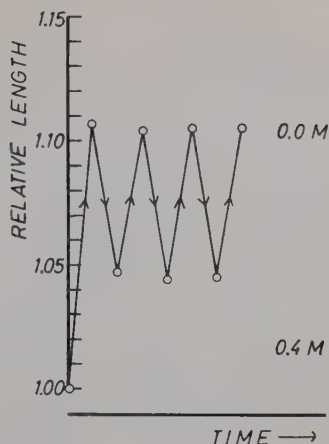
1. Determination of plastic and elastic component

With the aid of a core-borer and a razor-blade, circular discs about 20 mm. in diameter and 2 mm. thick, all of the same size, were cut out of a potato tuber. The discs, in this experiment 20 in number, were put alternatively in 0.40 *M* and 0.00 *M* mannitol solutions, being 10^{-5} *M* in respect to CaCl_2 and K_2SO_4 . If not otherwise stated all distilled water and mannitol solutions mentioned in the following are 10^{-5} *M* in respect to these salts. This in order to get a less poisonous medium for the tissue. The discs remained in each concentration for 4 hours after which time two diameters at right angles to each other were measured on each disc. The result is shown in Figure 1.

The plastic component is represented by the irreversible increase in length of the parenchyma when plasmolysed for a second time after full distension

Figure 1. *Plastic and elastic component of potato tuber parenchyma.* The ordinate shows the diameter length of parenchyma discs when alternately placed in 0.4 and 0.0 *M* mannitol for 4 hours. The standard error of the means did not exceed 0.006. It was calculated

from the formula $\sqrt{\frac{\sum(x - \bar{x})^2}{n(n-1)}}$, where x stands for individual values, \bar{x} for their arithmetic mean, and n for number of items; in this case $n=40$.



at maximum turgor pressure. The reversible change in length after such a treatment is called the elastic component. From this experiment one can see that no measurable plastic component persists after one complete distension and that the elastic component remains. Thus after complete turgor distension the parenchyma is in a state of perfect elasticity (cf. Brauner and Brauner 1943).

2. Determination of turgor pressure

For these determinations parenchyma discs were prepared in a similar way as for the determination of the plastic and elastic component. Five such discs were uniformly distributed into each of a number of mannitol solutions of different osmotic value. After 3 hours in the solution 2 diameters, at right angles to each other, were measured on each disc, in a dissecting microscope. In Figure 2 the average value of the diameters versus the osmotic value of the surrounding solution is seen. Incipient plasmolysis seems to occur in 0.4 *M* mannitol (cf. Hasman 1943, Meyer and Wallace 1941, and Oppenheimer 1930).

As the parenchyma of potato tuber is isotropic, *i.e.* the change in length of the parenchyma is the same in all directions, the change of volume can be calculated directly from the change in length. If it is assumed that the contents of the cell respond to Boyle—van't Hoff law, then the osmotic value of the cell contents can be calculated for each cell volume (Tamiya 1938). At equilibrium the suction force of the cell must be equal to that of the surrounding solution. The turgor pressure p , *i.e.* the excess hydrostatic pressure inside the cell, is thus the difference between the osmotic value of the cell contents, O_c , and that of the surrounding solution, O_e

$$p = O_c - O_e \quad (4)$$

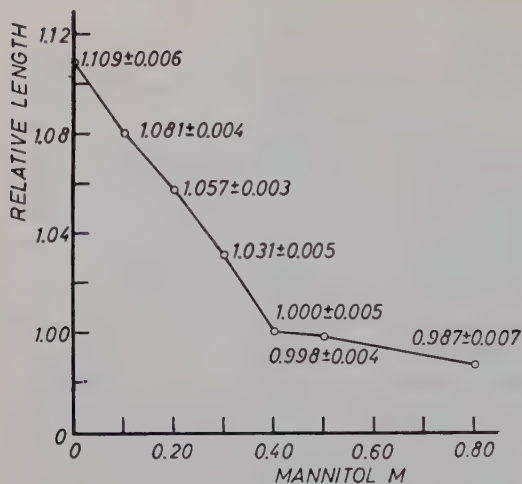


Figure 2. "Suction force curve" of potato tuber parenchyma. The ordinate shows the diameter length of parenchyma discs placed in mannitol solutions of different concentrations for 3 hours. The standard error of the means are shown in the Figure; $n=10$. The suction force of the fresh parenchyma corresponded to 0.3 M mannitol.

The relation between the osmotic values and the turgor pressure at different cell volume is shown in Figure 3.

3. Determination of resonance frequencies

With the aid of a double-edged razorblade knife pieces of parenchyma, $1.5 \times 1.5 \times 20$ mm. in size unless otherwise stated, were cut out from potato tubers. These pieces of parenchyma were put in a solution of distilled water, for at least one hour before further used.

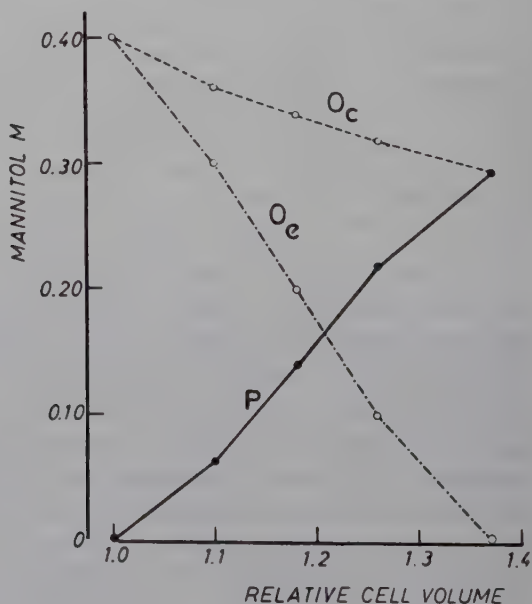


Figure 3. Inter-relationships between osmotic value of the cell contents (O_c), suction force (O_e), turgor pressure (p), and cell volume. Ordinate: atmospheres expressed as M mannitol.

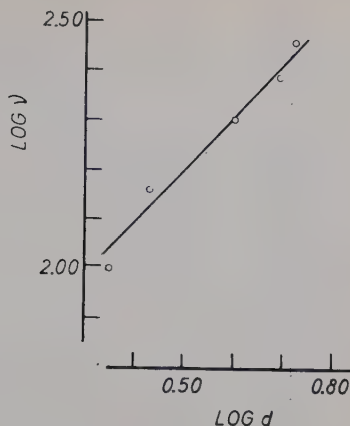


Figure 4. *Effect of the thickness (d) of the parenchyma on the resonance frequency (ν). The length of the material was 10 mm.*

The resonance frequency of potato tuber parenchyma at room temperature, 20—22°C, remains constant, *i.e.* the variations are smaller than 2 %, for a period of at least 8 hours and sometimes even for 24 hours in 0.00 *M* as well as 0.50 *M* mannitol, after which time the resonance frequency begins to sink. It is of no importance whether the material performs elastic vibrations all the time or only during the measurements.

A change in temperature to 5 or 30°C has no visible effect on the resonance frequency, but the constancy is not as good at higher temperatures as at room temperature.

Figure 4 shows the relation between the resonance frequency and the thickness of the material in the direction of vibration, when the length is kept constant. The resonance frequency is directly proportional to the thickness. The importance for the resonance frequency of the length of the plant material is shown in Figure 5. The resonance frequency is inversely proportional to the square of the length.

If one assumes that the resonance frequency is determined by the thickness d and the length l according to the following formula

$$\nu = K_1 \cdot d^a \cdot l^b \quad (1)$$

where K_1 , a , and b are constants, and this formula is logarithmed:

$$\log \nu = \log K_1 + a \cdot \log d + b \cdot \log l \quad (2)$$

then the relation between the logarithm of the resonance frequency and the logarithm of the thickness shall be a straight line with the slope a , when the length is kept constant. Also the corresponding relation as regards the length when the thickness is kept constant, shall be a straight line with the slope b .

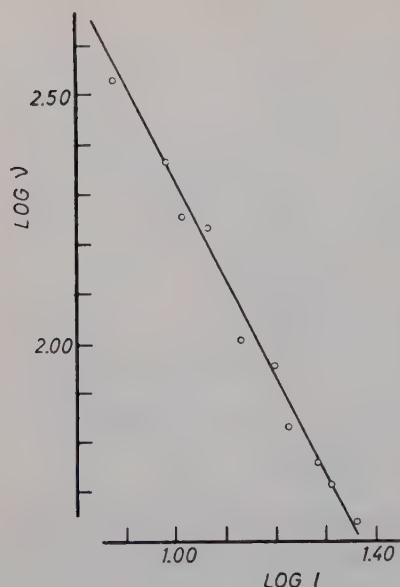


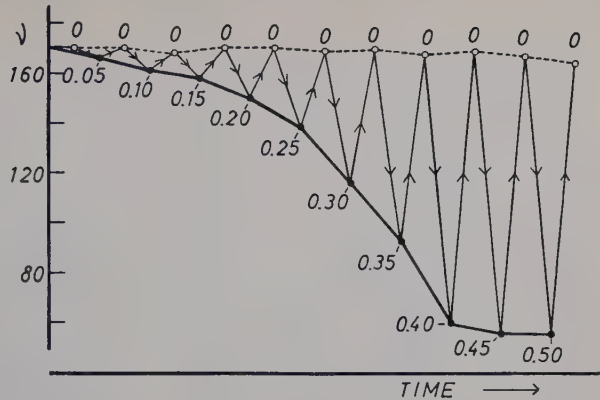
Figure 5. *Effect of the length (l) of the parenchyma on the resonance frequency (v). The thickness of the material was 4 mm.*

From the Figures 4 and 5 it is seen that these coefficients are +1 resp. —2. The original formula can thus be written

$$v = K_1 \cdot \frac{d}{l^2} \quad (3)$$

The osmotic value of the surrounding solution strongly influences the resonance frequency. The changes are quite reversible, however, so that as soon as a state of equilibrium is reached, every osmotic value in the surrounding solution corresponds to a certain resonance frequency of the plant material. Figure 6 shows the results from an experiment where the osmotic value of the surrounding solution has successively been increased every other time and every other time has been brought back to zero. The readings of the resonance frequencies have been made as soon as equilibrium has been reached in the new solution, which of course takes a longer time for greater changes in the osmotic value of the surrounding solution than for smaller ones. One can see how the resonance frequency decreases as the osmotic value of the solution increases, at first slowly, then faster, and at last suddenly becomes fairly stable. However at still higher osmotic values in the surrounding solution the resonance frequency decreases even more. The reversion of the resonance frequencies in the solutions with the osmotic value zero, is very good.

Figure 6. *The reversible changes of the resonance frequency (ν) of potato tuber parenchyma, when alternately placed in distilled water and mannitol solutions of increasing concentrations. Readings of resonance frequencies after osmotic equilibrium. Cf. Figure 1.*



To be able to directly compare different relations of resonance frequency versus the osmotic value of the surrounding solution (in the following called ν/O_e curves) the pieces of parenchyma must be exactly alike so that the resonance frequency of the different pieces in a 0.00 M solution is the same. This is very difficult to achieve in reality. It was shown above, however, the way in which thickness and length affect the resonance frequency (Figures 4 and 5). One can therefore choose a resonance frequency of *e.g.* 200 p/s in a 0.00 M solution as an arbitrary starting point and calculate the other resonance frequencies in relation to this value. By using the formula (3) it is possible to calculate the values for the thickness and length which the plant material should have in order to give a resonance frequency of 200 p/s in a 0.00 M solution. The other resonance frequencies which have been determined by experiments, can then be corrected in the same way taking a frequency of 200 p/s as a standard. Thus it is possible to compare different ν/O_e curves which can then be statistically handled.

Figure 7 shows a series of ν/O_e curves from parenchyma of one and the same potato tuber. The experiment has been performed with three different groups, each with 5 pieces, covering the ranges 0.00–0.30 M, 0.30–0.50 M and 0.50–0.80 M mannitol in the surrounding solution. The resonance frequency of the first group has in 0.00 M mannitol been corrected so as to have a value of 200 p/s. The second group has in 0.30 M mannitol been given a resonance frequency which corresponds to the average of the first group in this concentration. The third group has been treated in a similar way. In this manner it is possible to construct ν/O_e curves for a plant material over a broad range with great accuracy. The reason for using this method is also that the change of the resonance frequency caused by the change in the osmotic value of the surrounding solution is sometimes so great that the

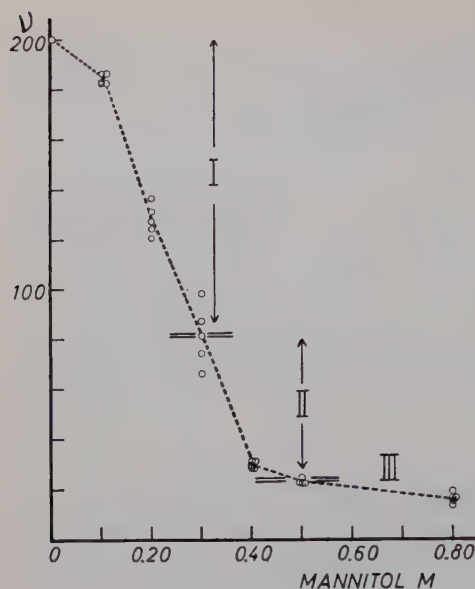


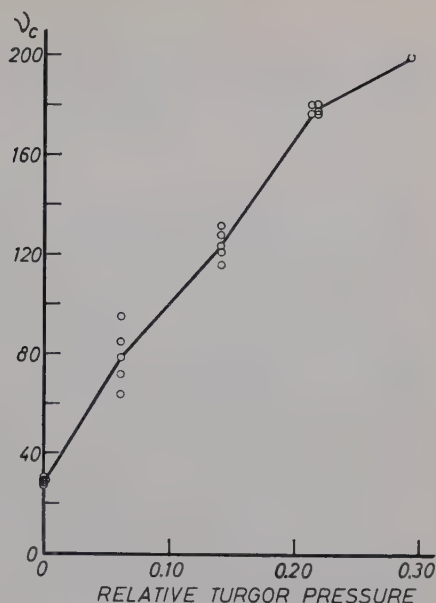
Figure 7. Series of $\sqrt{O_e}$ curves from parenchyma of one and the same potato tuber. Every curve (I—III) is the mean of five single curves. The starting point of curve I has arbitrarily been given the resonance frequency of 200 p/s, i.e. the values of the five single curves belonging to curve I have been mathematically corrected as to coincide at this value. In the same way the starting point for curve II coincides with the last value of curve I, and analogously for curve III.

values obtained for resonance frequency cannot in practice be measured with one and the same piece of plant material. This because the lowest frequency obtainable by means of the oscillator was 36 p/s. Therefore pieces of different length are used according to the range of concentration which is being investigated. It is thus always possible to use frequencies in the vicinity of 200 p/s, i.e. to use a frequency range within which the readings can be done in the easiest way, at least with the pieces of potato tuber parenchyma, which were used in this investigation.

Discussion

The relation between the resonance frequency and the osmotic value of the surrounding solution is seen in Figure 7. From the values shown in Figure 3 the turgor pressure can be determined as being a function of the osmotic value of the surrounding solution. By comparing these figures, one can obtain the resonance frequency as a function of the turgor pressure. However, this resonance frequency has to be corrected according to the change in thickness and length shown in Figure 2. These changes, however, have very little effect on the resonance frequency. As the resonance frequency is directly proportional to the thickness (Figure 4) but inversely proportional to the square of the length (Figure 5), the corrected value for the resonance frequency is only

Figure 8. *The relation between resonance frequency (ν_c) and turgor pressure. The frequency values have been corrected for the simultaneously occurring changes in thickness and length. The relative turgor pressure is expressed as M mannitol.*



inversely proportional to the length. This because the potato tuber parenchyma is isotropic, *i.e.* the relative change in size due to osmosis is equal in all directions. The different values of resonance frequency are thus calculated by the formula

$$\nu_c = \frac{\nu \cdot l}{l_0} \quad (5)$$

in which ν_c is the corrected value of the resonance frequency of a material with the length l . l_0 is the length of the same material in a solution with the osmotic value zero.

Figure 8 shows how the corrected resonance frequency depends on the turgor pressure. From the figure is evident that it is possible from such a "calibration curve" to calculate the turgor pressure of the investigated material by measuring the corresponding resonance frequency.

One can compare the vibrating pieces of parenchyma with a vibrating rod clamped at one end. The following formula is valid for the resonance frequency in vacuum of the latter (Bergman and Schaefer p. 405, 1954):

$$\nu = \frac{s^2 \cdot d}{4 \pi \cdot l^2 \cdot \sqrt{3}} \cdot \sqrt{\frac{E}{\rho}} \quad (6)$$

in which s is a constant, giving ν for the fundamental resonance frequency or for any higher harmonic, d is the thickness of the rod in the direction of

vibration (its width is of no importance), l is the length, E is Young's modulus, and ρ the density of the rod. Young's modulus E is defined as follows (Bergman and Schaefer p. 204, 1954):

$$E = \frac{l \cdot F}{\Delta l \cdot q} \quad (7)$$

where Δl is the change of the length l of a rod with a cross section q , when stretched by a force F . It follows, that a body with a high elasticity has a low Young's modulus and *vice versa*.

Figure 7 makes clear, that the great changes which take place in resonance frequency in solutions of different osmotic value are only to a minor part due to the changes in thickness and length caused by osmosis. Nor can the changes in density, which are very small alone or even together with the changes in thickness and length, account for the different resonance frequency. Thus the only remaining explanation is that Young's modulus of the whole parenchyma is changing due to the different turgor pressure. By taking the square of the above formula (6) and correcting in regard to changes in thickness and length, the formula

$$v^2 = K_2 \cdot E \quad (8)$$

is obtained, in which K_2 is a constant. Thus one should be able to calculate E as a function of turgor pressure (see Figure 4 in Nilsson *et al.* 1958) and compare it with the value for E obtained by other experimental methods so as really to be able to state that the changes in resonance frequency are mainly due to changes in E . However, the determinations of the resonance frequencies had to be carried out under such conditions, that the formula (6) could not be used directly as such when determining Young's modulus E of the whole parenchyma. Below follows a discussion as to which conclusions can be drawn concerning the change of Young's modulus with the turgor pressure, in consequence of the experimental results.

The fact that the formula (6) is valid for an oscillating rod in vacuum only, and that the experiments were performed in a mannitol solution makes it impossible to calculate E directly from formula (6). If the rod oscillates in a liquid, the friction experienced by the rod in the surrounding solution results in a decrease of the resonance frequency. In the following it will be shown, that E as calculated from formula (6) differs from the correct value of Young's modulus by a constant R , the magnitude of which depends only on water friction.

The differential equation for a vibrating rod in vacuum is given by

$$\rho \cdot q \cdot \frac{d^2 y}{dt^2} + E \cdot I \cdot \frac{d^4 y}{dx^4} = 0 \quad (9)$$

where ρ is the density of the rod material, q its cross section, I its moment of inertia and y the amplitude of the vibrating rod at a distance x from its clamped end (Weizel, p. 203, 1949). If the friction force experienced by each element of length, dx , of the rod is proportional to the velocity $\frac{dy}{dt}$ of this element the formula (9) takes the form

$$\rho \cdot q \cdot \frac{d^2 y}{dt^2} + R' \frac{dy}{dt} + E \cdot I \cdot \frac{d^4 y}{dx^4} = 0 \quad (10)$$

The solution of this equation can be written as

$$y(x, t) = f(x) e^{-kt} \cos 2\pi \nu t \quad (11)$$

from which the resonance frequency in the fundamental vibration can be derived by using proper boundary conditions. The result is

$$E = \frac{12 \rho \cdot I^4}{s^4 \cdot d^2} (4\pi^2 \nu^2 + \kappa^2) = E' + R \quad (12)$$

E being the true Young's modulus and $R = \frac{12 \rho I^4}{s^4 \cdot d^2} \cdot \kappa^2$. This implies that the relative change of E with turgor pressure is equal to the relative change of E' calculated from formula (6), if the friction force really is proportional to the velocity $\frac{dy}{dt}$ of the rod. But this is the case in the above named experiments since there is no turbulence (Bergman and Schaefer 1954, p. 254). Further, since the water friction is relatively small, formula (12) can be used also for the resonance frequency of forced oscillations, although the above cited derivation only applies to free vibrations.

It seems to be easy to determine R experimentally once and for all by measuring the resonance frequency of a strip in air and in mannitol solution. This determination, however, turns out to be difficult because of the water adhering to the strip when removed from the solution. But even if such a measurement could be done, a calculation of E from the results presented above would not be possible, since in the experiments a small iron spiral had to be clamped to the top of the strip. This additional mass decreases the resonance frequency. For the simple case of a rod with a point mass, m_2 , at the top of the rod the resonance frequency is given by

$$\nu^2 = \frac{E \cdot I}{4\pi^2 I^3 \left(\frac{m_1}{s^4} + \frac{m_2}{3} \right)} \quad (13)$$

where m_1 is the mass of the rod and $I = \frac{d^4}{12}$ its moment of inertia in our case.

If $m_2 = 0$ this formula reduces to formula (6). It is readily seen from this formula that the additional mass m_2 increases the effective mass of the vibrating rod. Since the mass m_2 , which in our case was about 2 mg, was distributed

along the upper part of the rod, this formula cannot be used for the exact determination of E and R , but it is possible to draw the conclusion, that E must be proportional to v^2 .

If v^2 is plotted versus the turgor pressure, strong evidence is obtained that v^2 and thus E is proportional to the turgor pressure. Figure 4 in Nilsson *et al.* (1958) shows this dependence. The E values given at the ordinate are calculated by using the formula (13) with $l=0.7$ cm, $d=0.15$ cm, and $m_2=2$ mg.

While the resonance frequency method described above allows a good determination of the dependence of Young's modulus on turgor pressure, the absolute values of E are more easily obtained by using the conventional method for the measurement of Young's modulus (cf. *e.g.* Brauner and Brauner 1943). On the other hand, because of experimental difficulties, the results yielded by this method are not exact enough to prove that Young's modulus is proportional to the turgor pressure. Since for the sake of theory presented in Nilsson *et al.* it is necessary to know the absolute value of Young's modulus too. Therefore the following measurements of Young's modulus of potato tuber parenchyma were made.

III. Determination of Tissue Rigidity

Material and method

The parenchyma, which was used for these measurements of E , had the shape of parallelepipeds, 7 cm. long and with a square cross section, the size of which was varied. These pieces were treated in the same way as the material used for the measurements of the resonance frequency. The wanted turgor pressure of the cells was obtained by different concentration of mannitol in the surrounding solution. When osmotic equilibrium was reached, the pieces were taken out from the solution and immediately dipped in paraffin-oil to prevent osmotic changes during the following measurements, which were carried out in the open air. The pieces were hung vertically by clamping the top of the pieces in a screw-vice. In the lower end a piece of copper wire was fastened in such a way that different weights could be attached. On the centre of the piece of parenchyma and between the upper and lower fixations a definite section, about 4 cm. was marked out by two extremely thin pieces of copper wire, which were stuck into the parenchyma. The length of this section was measured by means of a cathetometer as soon as loads of different weights had been attached to the lower end. After each test the load was removed and the original length was checked. The experiment was interrupted when the original length was found to have changed, *i.e.* when the elastic limit evidently had been surpassed (Overbeck 1934, Brauner and Brauner 1943). The experimental errors of this method are of the same size as the biological variations of the material used.

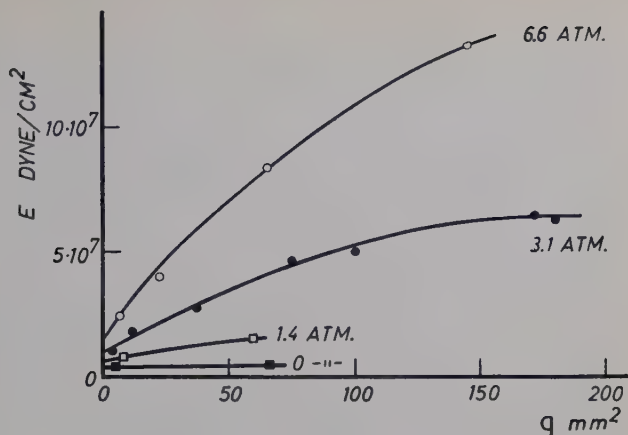


Figure 9. Young's modulus (E) of potato tuber parenchyma at different turgor pressures versus cross section (q).

Results

As is evident from the formula (7), E can be determined for a definite turgor pressure, if the quantities F , q , l , and Δl are known. Using the above mentioned method, Young's modulus was determined for different values of the cross section q and the turgor pressure p . The results are given in Figure 9 which shows a decided dependence of E on q , except in the case when the turgor pressure is zero.

Discussion

In the theory given in Nilsson *et al.* it will be shown, that it is possible to calculate Young's modulus of the parenchyma using a simplified model of the parenchyma. The theoretical results agree very well with the experimental values of E found for thin parenchyma strips. According to the theory E should be independent of the cross section of the strip as required by Hooke's law. Since this is not confirmed by the experiments some extra factor coming into play for large cross sections has obviously not been taken into account by the theory.

Now, while the cells of the parenchyma model used for the calculation are regular polyhedra and of the same size and shape, the cells in real parenchyma are of irregular and widely differing geometrical shapes. The cause for the difference may therefore be found in additional shearing stresses that are compelled to arise in this irregular tissue, when the parenchyma is subjected to a change of its geometry. Such a change can be brought forward either by applying an outer force or changing the turgor pressure inside the cells. The internal stress induced in the parenchyma because of this reason

will naturally increase with increasing cross section and turgor pressure. In both cases the pressure exerted on the cells near the center of the strip by the adjacent cells makes the cells to lose their individual freedom of movement the more the turgor pressure and cross section are increased. This explains the experimental result, which shows no dependence on q for $p=0$ (cells loosely linked to each other with plenty of room for individual movements), while for $p > 0$, E increases with q . It will be shown below that the following approximation holds true

$$E = E_{\text{ideal model}} + E_{\text{internal stresses}} \quad (14)$$

where the first term of the sum is calculated in Nilsson, Hertz and Falk (1958) for an ideal model deficient from internal stresses due to irregularities in the cell shapes. The second term corresponds to internal stresses which arise in real parenchyma if $q > 0$, its magnitude increasing with the cross section, q .

The determinations of the absolute values for Young's modulus were carried out in air with the aid of paraffin-oil to prevent changes in the osmotic conditions of the parenchyma due to evaporation. In this way volume changes of the cells during the stretching could not take place. This could be the case if the determinations had been carried out with the parenchyma immersed in a solution (Overbeck 1934). The reason for this way of measuring is that the values for Young's modulus obtained from these determinations is to be compared with the Young's modulus of a vibrating rod of parenchyma and also with the Young's modulus calculated in Nilsson *et al.*, based on models of cells with constant volume. In case of a vibrating rod of parenchyma no change of cell volume due to water permeation has time to occur.

IV. General Discussion

As shown in Figure 1 the potato tuber parenchyma seems to behave perfectly elastic after complete turgor distension. This explains the great reproducibility of the resonance frequency in solutions of different osmotic value (cf. Figure 6). Since the turgor pressure at equilibrium thus must have a value dependent only on the osmotic value of the surrounding solution, every resonance frequency corresponds to a certain turgor pressure. And conversely, if a "calibration curve" for the material under investigation is obtained, one can calculate the turgor pressure from the resonance frequency (cf. Figure 8). The reproducibility of the resonance frequency, as dependent on turgor pressure, also allows very good determinations of water permeability, repeated many times with the same sample of material (Virgin 1955).

The experimental results given in this paper and the theoretical results in Nilsson *et al.* seem to agree very well. That would thus indicate, that the premises of the parenchyma model for these studies are right. The cell wall material itself is believed to follow Hook's law, *i.e.* there are no changes in its elasticity during the experiments (Preston 1955). Nevertheless there are changes in the elasticity of the whole parenchyma due to turgor pressure, which, however, are reversible thanks to the ideal cell wall material. These changes are the cause of the different resonance frequencies shown in Figure 8. The dependence of E on turgor pressure p is found to be a straight line, the slope and intersection point of which can be calculated.

Summary

The rigidity of potato tuber parenchyma and its dependence on turgor pressure has been studied experimentally by the aid of two methods, one by measuring resonance frequencies and the other by direct determinations of Young's modulus. The results from these methods agree very well and also with the theoretical results given in Nilsson *et al.* They all indicate a linear dependence of Young's modulus (corrected for water friction and internal stresses etc.) on turgor pressure.

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On the Relation between Turgor Pressure and Tissue Rigidity. II

Theoretical Calculations on Model Systems

By

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I. Introduction

In the first part of this investigation (Falk, Hertz and Virgin 1958, in the following referred to as I) measurements on potato tuber parenchyma have been presented which indicate a linear dependence of Young's modulus on the turgor pressure in the cells of the parenchyma (see Figure 4). The purpose of the present paper is to show that this simple relation between elasticity and turgor pressure in the homogeneous cell-structure of the potato tuber can be explained by a simple model in which the liquid-filled cells are assumed to be bounded by thin elastic membranes. With certain additional assumptions this model permits the calculation of Young's modulus and its dependence on turgor pressure. The theoretical formula is shown to be in reasonably good agreement with the experimental results for thin strips of parenchyma.

Briefly, the essential features of the mechanism considered here are the following. The elasticity of the cell walls is recognized as the main factor responsible for the elasticity of plant tissues (cf. Frey-Wyssling 1952, p. 194). The role of the cell sap is then only an indirect one, but is nevertheless important in two respects. First, being incompressible, the fluid cell-content ensures that the volumes of all cells remain constant when a piece of the plant tissue is stretched. Secondly, the sap exerts a pressure on the cell-walls

(turgor pressure), causing them to be distended in a state of elastic stress, even when no external forces act on the tissue. These two effects will be decisive for the elastic properties and their dependence on the turgor pressure.

For the benefit of readers not interested in the mathematical details of the theory, its general assumptions, results and their comparison with experiment will be presented first.

II. Model of the Parenchyma

To make a mathematical treatment feasible we shall consider a simplified model of the potato tuber parenchyma, such that the cell walls are pictured as thin elastic membranes. Each cell is filled with an incompressible fluid, which exerts a pressure p in excess of the external atmospheric pressure on the cell walls. This pressure, which corresponds to the turgor pressure in the cells of the parenchyma, is supposed to be equal in all cells of the model. It is further assumed that the cell fluid cannot penetrate the walls when the parenchyma is being stretched.

More correctly, to represent the effect of the cytoplasm the model cell-walls have to be assumed semipermeable. But during the rapid deformations accompanying stretching they can certainly be regarded as quite impermeable.

When no external forces act on the parenchyma, the simplest picture of a typical cell in such a model is that of a sphere. If all cells were ideal spheres with the same radius r , the cell-structure could be that shown in Figure 1, where each sphere is supposed to be in contact with six neighbours (cubic packing). Denser packings would also be possible: *e.g.*, each sphere could touch twelve others. In either case the space between the spherical cells (which is not filled with fluid) would be much exaggerated as compared with the intercellular spaces in the real parenchyma of the potato tuber. Therefore a spherical model is only to be regarded as a simplified representation of the individual cells, not of the way in which they hold together to form a connected structure. In spite of this we shall continue to refer to the simple structure of Figure 1 for illustrative purposes. More realistic cell-structures will be considered later.

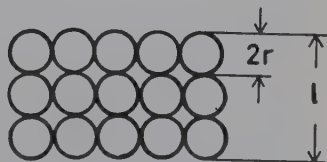


Figure 1. *Simplest model of potato parenchyma.* The spherical cells are bounded by elastic cell walls and filled with liquid, leaving air-filled interstitials between them.

The cell-wall material is supposed to follow Hooke's law of proportionality between stress and strain in small deformations. Thus, if a plane strip of a cell wall of length a and width b (cross-section $q=bd$, d being the constant thickness of the wall) is stretched in the a -direction by a force F , the reversible elongation Δa is taken to be of the form

$$\frac{\Delta a}{a} = \frac{1}{E_c} \frac{F}{q} = \frac{1}{C} \frac{F}{b}. \quad (1)$$

E_c is Young's modulus of the cell-wall material, and $C=E_c d$ may be called Young's "surface modulus" of the cell-wall. To characterize the elastic properties of the walls we need one more elastic constant, the Poisson coefficient μ , which describes the transverse contraction accompanying the elongation (1):

$$\frac{\Delta b}{b} = -\mu \frac{\Delta a}{a}. \quad (2)$$

In speaking of the elasticity of plant material, as we have done, some caution is necessary, for if a piece of such material is subjected to stress, its behaviour will be partly plastic and only partly elastic. We assume throughout that these different effects have been separated and that it is thus possible to concentrate attention on the elastic effects alone. Even so it may sometimes be impossible to define elastic constants in a strictly reproducible manner. All such difficulties are disregarded in our model.

Further, a relation of the form (1) with a unique constant C is certainly an oversimplification of the real properties of the cell-wall, and does not account for the fact that the submicroscopic wall texture is neither homogeneous nor isotropic. However, in, for instance, potato tuber parenchyma the cells are oriented at random; and a possible anisotropy in the individual walls should not then be important for the over-all elastic properties of pieces containing many cells.

III. Theoretical Results

Determination of the cell-wall constants

Let us think of the parenchyma as the spherical structure of Figure 1. If the excess pressure of the cell fluid, *i.e.* the turgor pressure, is increased in each cell from 0 to p , the radii of all the cells will increase from, say, r_0 to r , and thus the dimensions of the parenchyma-model will also increase. (Note that we always mean reversible changes.) The relative increase $(l-l_0)/l_0$ of the length of the strip is equal to the relative increase $(r-r_0)/r_0$ of the cell radius, which in turn should be proportional to the pressure. A closer investigation gives the approximate relation (cf. eq. (20))

$$\frac{l-l_0}{l_0} = \frac{r-r_0}{r_0} = \frac{1-\mu}{C} \frac{pr_0}{2} = \frac{1}{D} \frac{pr_0}{2}$$

or

$$\frac{D}{r_0} = \frac{p}{2} \frac{l_0}{l-l_0}, \quad (3)$$

where we have written D for the combination $C/(1-\mu)$. With an appropriate definition of the cell "radius" this formula is correct even for the more complicated cell-forms discussed later.

Measurements of the dependence of the cell radius on turgor pressure have been made in part I (Falk, Hertz and Virgin 1958) and the linear relation predicted by theory has been confirmed by experiment as long as no plastic changes take place in the cell material.

In the experimental procedure used in I a parenchyma sample is placed in manitol solutions of different concentrations. Since it was established that, for reasons similar to those discussed below, the relative changes in the dimensions of the sample depend on its geometry, we determined D/r_0 with the help of thin strips of parenchyma rather than use the results obtained with cylindrical disks in I. When the length of these strips was measured at turgor pressure 0 and 6.5 atm., $(l-l_0)/l_0$ was found to be about 0.15 (the cross-section of the sample being about 10 mm²). Using these values and expressing p in dynes per cm², we get from (3)

$$\frac{D}{r_0} = 2.1 \cdot 10^7 \text{ dyne/cm}^2$$

and, as the average radius r_0 was determined to be 0.05 mm,

$$D = \frac{C}{1-\mu} = 1 \cdot 10^5 \text{ dyne/cm}$$

(1 atm. $\approx 10^6$ dyne/cm², 10^6 dynes being approximately the weight of 1 kg).

The individual constants C and μ cannot be obtained by this method. However, since the determination of *e.g.* Poisson's constant μ for the cell-walls proves to be difficult, and the knowledge of its exact magnitude will be of little importance for our purposes, it was taken to be about $1/3$. This value is in good agreement with the μ -values of other materials and should be correct to within ± 30 per cent.

Calculation of Young's modulus

Suppose a strip of the parenchyma or of the model is available, of length $l \gg 2r$ and cross-section $q \gg \pi r^2$. If Hooke's law is valid here, the reversible elongation Δl on exposing the strip to a tensile force F in the direction of l should be of the form

$$\frac{\Delta l}{l} = \frac{1}{E} \frac{F}{q} \quad (4)$$

(cf. eq. (1)), E being now Young's modulus of the parenchyma. It will be shown below that the model does indeed follow Hooke's law and, with some additional assumptions, allows the determination of E .

In this calculation we are not going to utilize eq. (4) as it stands, but in an equivalent form involving the notion of energy or work. The general course of the argument is this. When the strip is stretched, the individual cells in it will be elongated in the direction of the force. Thus the form of the cells in the model of Figure 1 will no longer be spherical, but *their volumes must remain constant* because the incompressible cell fluid cannot penetrate the walls. Since, of all geometrical forms, the sphere has the least surface for a given volume, the surface of each cell must be increased when the force F is applied. This means that additional potential energy ΔU is stored in the elastic cell walls. Knowing the elastic constants of the walls, it should be possible to calculate ΔU for a given deformation, *e.g.* by first treating one typical cell and then multiplying by the number of cells in the strip.

On the other hand the increased energy is equal to the work W performed by the applied force when stretching the strip. It is a direct consequence of eq. (4) that

$$W = \frac{1}{2} \frac{Eq}{l} \Delta l^2 = \frac{1}{2} EV \left(\frac{\Delta l}{l} \right)^2 \quad (5)$$

$V=ql$ being the volume of the strip. Identification of (5) with the expression calculated for ΔU will yield Young's modulus E of the cell-structure.

In the actual calculation given below we have in mind cells whose walls are completely grown together with those of contiguous cells. The average deformation (eq. (26)) will then be such that a spherical cell would go over into an ellipsoid of revolution with its axis pointing in the direction of the applied force (see Figure 2). It is not difficult to calculate the work necessary for such a deformation. If the number of cells in the parenchyma strip is supposed to be given by the ratio of the strip volume to the cell volume, the resulting expression for Young's modulus is found to be approximately (see eq. (31))

$$E = \left[1 + \frac{7-5\mu}{20(1+\mu)} \right] 3p + \frac{3(7-5\mu)}{10(1+\mu)} \frac{D}{r_0}. \quad (6)$$

It should be mentioned, however, that in a strictly spherical model the unavoidable intercellular spaces will reduce the possible number of cells in a given strip volume. For the cubic packing of Figure 1, this effect introduces a factor $\pi/6=0.524$ multiplying the whole expression (6) for E . But again this structure, in which the cell walls do not adhere to each other except at isolated points, is not to be taken literally.

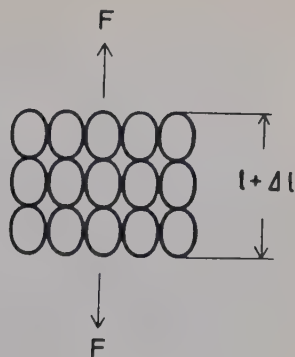


Figure 2. *Parenchyma model under stress.* Each cell is deformed into an ellipsoid, its volume being constant because of the incompressible cell fluid. To attain this deformation the surface of each cell must be increased, i.e. the cell wall be stretched.

In accordance with the experimental results presented in I, E is a linear function of the turgor pressure. It is of special interest that the p -dependent term does not involve cell constants such as C or r_0 . It does involve μ , but if μ ranges from 0 to 0.4 the value is changed by less than 20 per cent. Since μ is certainly greater than 0 and less than 0.4, this means that a check of the theory by experiment should be possible, almost independently of the accuracy with which the properties of the cell walls have been investigated, by studying the increase of E with turgor pressure. Using the values of D/r_0 and μ given in the preceding section, both terms in the expression for E can be evaluated to give

$$E = 3.6p + 2.5 \cdot 10^7 \text{ dyne/cm}^2. \quad (7)$$

To obtain E in dynes per cm^2 , p should be expressed in the same unit.

Remark on different cell-forms

The assumptions for the spherical model above being somewhat inconsequential, it is desirable to check the result (6) or (7) by using more consistent variants of the model. Let us imagine a regular cell-structure built up of equal isodiametric polyhedra filling the space compactly. It turns out that Young's modulus of such a structure will be slightly sensitive to the assumed cell-form and also to the direction of the applied force. As an example we choose the case of tetrakaidecahedral cells. Each cell is then assumed to be a polyhedron bounded by fourteen faces (Figure 3 A), which very nearly represents the "ideal" cell-form (cf. Frey-Wyssling 1952, p. 195, Lewis 1946, Thomson 1887). If the force acts in the direction indicated as (a), we get (eq. (33 a))

$$E = 3.63p + 2.7 \cdot 10^7 \text{ dyne/cm}^2,$$

and if the direction is that of (b) (eq. (33 b))

$$E = 3.50p + 2.1 \cdot 10^7 \text{ dyne/cm}^2.$$

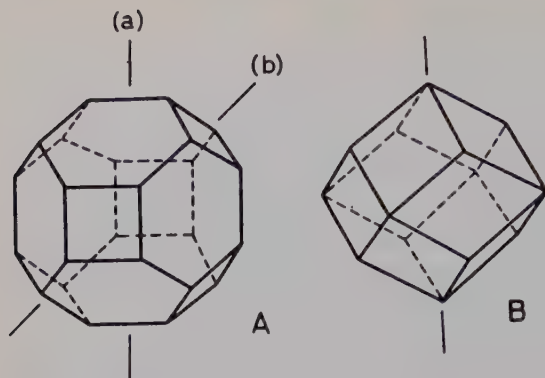


Figure 3. *More realistic cell forms:*
A) *isodiametric tetrakaidecahedron*
and B) *rhombic dodecahedron*.

Similar deviations from (7) are found for other cell-forms; they are seen not to be very important, especially in view of other uncertainties inherent in the model and mentioned in the next section. Moreover, it is shown in the mathematical part below that, for any of the structures considered, the value of E , averaged over all possible directions of the force, is always given by (7), or more generally by (6). In the case of cells of arbitrary shapes and oriented at random this will therefore be the expression most nearly justified on the basis of the simple model developed so far.

Modifications of the formula for E

In this section two effects tending to modify the above expression for E will be mentioned. The first, which serves to decrease both terms in the expression for E , has the following background. In the calculation leading to eq. (6) the cell-structure was assumed to undergo a certain uniform deformation on stretching, but in reality this will not be quite true. The theoretically correct deformation of all individual cell-walls for a given elongation of the strip could in principle be obtained from the condition that the total increase ΔU of the elastic energy stored in them should be as small as possible. Now the cell-walls do not adhere to each other along the intercellular spaces; therefore, they have a greater freedom than supposed above for adjusting themselves so as to keep down the value of the elastic energy. As a result ΔU will be smaller than before, and so E , being proportional to ΔU , will be smaller too.

We will not attempt to evaluate this effect for a realistic cell-structure, which would not be very easy. But an estimate of the order of magnitude can be formed by considering the case of spherical cells. Suppose such a cell is deformed into ellipsoidal shape as before (Figure 2), but without any restric-

tions on the internal displacements along the surface. The resulting expression for E is given by eq. (38), or using the experimental values above and again neglecting the volume of the intercellularies:

$$E = 1.8p + 0.63 \cdot 10^7 \text{ dyne/cm}^2.$$

Note that for the spherical model of Figure 1 this result has again to be multiplied by 0.524, besides being further decreased because of the freedom of the cells to be deformed to other shapes.

Comparing with eq. (7) we see that Young's modulus has been decreased considerably as a consequence of the extra freedom accorded to the walls. However, the walls are certainly not free to the extent assumed here, and it is argued below that a more probable reduction of (7) will be, roughly,

$$E = 3p + 1.5 \cdot 10^7 \text{ dyne/cm}^2. \quad (8)$$

Both terms, and especially the second, are rather uncertain.

The second effect is concerned with the elastic behaviour of the cell walls, which — even if it be supposed well defined — need not be exactly Hookean, *i. e.* the linear relation (1) between stress and strain may have to be supplemented by quadratic (and higher) terms. Although the presence of such terms will not make itself felt in infinitesimal deformations of the walls, it will have a significant bearing especially on the p -dependent term in the expression for E . (For that reason the mathematical formalism to follow will be developed in somewhat greater generality than indicated in the broad outline of the theory presented above.) As we do not know the exact elastic properties, there must be some ambiguity in the theoretical predictions. For instance, if the submicroscopic texture of the cell wall leads to a decreasing extensibility with increasing stress or wall pressure (*cf.* Frey-Wyssling 1952, p.222), the p -dependence will probably be more pronounced than in the formulas given above.

Finally, it should be pointed out that, apart from the effects mentioned in this section, the linear relation between E and p is only a first approximation and cannot be expected to hold good for too high values of p . (The p -dependence is an expression of the fact that it is *more difficult to deform a cell whose walls are kept distended*. The extra work required will be approximately — but only approximately — proportional to the stress in the walls, *i. e.* in our case to p .)

IV. Comparison with Experiment

It was shown experimentally in I that parenchyma strips of different cross-sections and immersed in the same mannitol solution do not exhibit the same value of Young's modulus. This behaviour would seem to contradict Hooke's law. It should be remembered, however, that the relation (4) or (5) pre-supposes a homogeneous material of quite definite internal properties. Neither of these conditions is fulfilled in this case. The osmotic equilibrium will not

correspond to an exactly homogeneous state, and the irregular geometrical configuration of the individual cells will introduce extra internal stresses in the parenchyma, different for different cross-sections. These effects have not been taken into account in the parenchyma-model discussed above, and will not be further investigated here. Since they should be more pronounced for thick pieces, the theory outlined above can be checked only by comparison with experimental results obtained from very thin strips of parenchyma.

The resonance frequency method proposed by Virgin (1955) has been proved superior to the conventional method of determining Young's modulus, if relatively small samples of biological material are concerned. But for the special case of potato tuber parenchyma this method suffers from the drawback that the resonance frequency has to be determined while the potato strip is submerged in a mannitol solution of known osmotic pressure. As pointed out in I, this adds a certain term to the value of Young's modulus determined from the formula for the resonance frequency in vacuum of a rod clamped at one end. This term R is due to the friction in water that is experienced by the oscillating potato strip (and also to internal friction). Since R cannot easily be determined with desired accuracy, this method is not suitable for checking the term independent of p in eq. (8). On the other hand, using the experimental results and eq. (6) of I, it can be shown that within experimental error E really is a linear function of the turgor pressure p (cf. Figure 4). The p -coefficient can be read off from the slope of the line in Figure 4 and is found to be 3.5, in good agreement with the theoretical value of about 3.

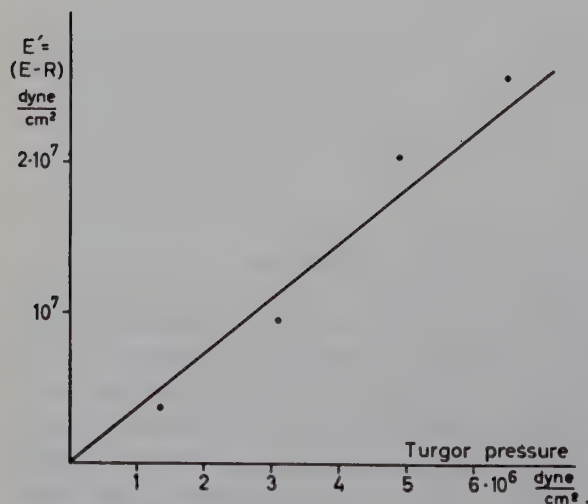


Figure 4. Dependence of Young's modulus E on turgor pressure p as determined from the experimental results given in I (Figure 8). (Apart from a term R due to water friction.)

The constant term in the expression (8) for E can be checked by comparing it with the results presented in I, where Young's modulus of a thin parenchyma-strip was measured by a conventional method. For $p=0$, E was experimentally found to be $0.5 \cdot 10^7$ dyne/cm², which is at least of the same order of magnitude as the approximate theoretical value $1 \cdot 10^7$ or $2 \cdot 10^7$ dyne/cm² given in (8). The discrepancy may be partly explained by variations in the potato tuber material used in the different experiments.

The fairly good agreement between the theory outlined above and the experimental values for E and its dependence on turgor pressure makes us believe that the mechanism proposed in this paper is the main factor responsible for the mechanical properties of thin samples of potato tuber parenchyma and similar tissues.

V. Mathematical Treatment

General considerations. Mathematical model

The theoretical discussion of the elastic properties will now be resumed in greater mathematical detail. First let us recall the main line of argument. On stretching a bar or strip of material following Hooke's law from a length l to $l+\Delta l$, the potential energy stored in a volume V is increased by

$$\Delta U = \frac{1}{2} EV \left(\frac{\Delta l}{l} \right)^2, \quad (9)$$

where E is Young's modulus (cf. eq. (5)). If the material has cellular structure, as in the case of potato tuber parenchyma, this energy is to be identified with the increased elastic energy of the cell-walls, which have been deformed in the course of stretching. It should therefore be possible to determine E in terms of quantities representing the state and properties of the walls, provided we can obtain an expression for the total wall energy as a function of the elongation Δl of the whole piece.

To be able to do that we must assume that the cell-structure has certain simple properties; and so we are really going to consider the behaviour of an idealized model, supposedly realistic enough for some of the salient features of the actual parenchyma to be reproduced. The most important simplifying assumptions that we shall make are these:

- (a) The cell walls are homogeneous, isotropic, and elastic, and thin enough not to offer any resistance to pure bending (membrane approximation).
- (b) In the absence of external forces, all cell walls are free from internal stresses when the turgor pressure of the cell fluid is zero.
- (c) All cells are of equal size and form, with three equal diameters. They are arranged to form a regular structure.

To complete the description the assumed form and arrangement of the cells should also be stated, but this question will not be important till the final steps of

the calculation and may be left open for the present. Even the formulation of assumption (c) above, which implies (b), is too restrictive for most of the following considerations; altogether, a more precise meaning will be given to our assumptions as the need arises.

According to (a) we may regard the cell walls in the model as ideal surfaces, whose energy content is completely determined by the state of surface strain. This state is conveniently represented by a strain tensor in the following way. Imagine a network of parameter curves drawn on the surface and moving with it, so that the same pair of parameters (u_1, u_2) is always attached to the same material point. Let the parameter values of two arbitrary neighbouring points differ by du_1, du_2 ; then, if the squared distance between them is written

$$ds_0^2 = g_{11}du_1^2 + 2g_{12}du_1du_2 + g_{22}du_2^2 \equiv \Sigma g_{ik}du_i du_k$$

on the unstrained surface and

$$ds^2 = G_{11}du_1^2 + 2G_{12}du_1du_2 + G_{22}du_2^2 \equiv \Sigma G_{ik}du_i du_k \quad (10)$$

after straining, the symmetrical strain tensor is defined to have the components

$$\gamma_{ik} = \frac{1}{2} (G_{ik} - g_{ik})$$

(functions of u_1 and u_2).

For our present purpose it will not be necessary to use the apparatus of tensor algebra. The original network on an unstrained cell surface may be chosen to be orthogonal ($g_{12}=0$), and only such deformations will be considered below in which it remains orthogonal ($G_{12}=0$). In that case $\gamma_{12}=0$, and the state of strain is characterized by just two functions γ_{11} and γ_{22} . The combinations

$$\gamma_1 = \gamma_{11}/g_{11}, \quad \gamma_2 = \gamma_{22}/g_{22}$$

have an immediate geometrical significance (and this holds also for $\gamma_{12} \neq 0$). In the strained state the distance between two neighbouring points on the curve $u_2 = \text{const.}$ is, using the definitions above,

$$ds_1 = \sqrt{G_{11}} du_1 = \sqrt{g_{11} + 2\gamma_{11}} du_1 = \sqrt{1 + 2\gamma_1} ds_{10},$$

where $ds_{10} = \sqrt{g_{11}} du_1$ is the distance between the same material points before straining. Consequently, the relative elongation in the u_1 -direction is given by

$$\frac{ds_1 - ds_{10}}{ds_{10}} = \sqrt{1 + 2\gamma_1} - 1 \approx \gamma_1.$$

Similarly for γ_2 .

The elastic surface energy is the quantity of present interest. It can be expressed as a sum or integral, extended over all cell walls,

$$U = \int w dS_0, \quad (11)$$

where $dS_0 (= \sqrt{g_{11}g_{22}} du_1 du_2)$ is an element of area on the unstrained surface. The functional dependence of the energy density w on the strain determines the elastic properties of the idealized cell wall (cf. *e.g.* Green and Zerna 1954, Ch. II, XII). For instance, the components of physical surface stress (force across unit length on the strained surface) can be expressed in terms of the strain as

$$\tau_1 = \frac{\lambda_1}{\lambda_2} \frac{\partial w}{\partial \gamma_1}, \quad \tau_2 = \frac{\lambda_2}{\lambda_1} \frac{\partial w}{\partial \gamma_2}. \quad (12)$$

where $\lambda_1^2 = G_{11}/g_{11} = 1 + 2\gamma_1$, $\lambda_2^2 = G_{22}/g_{22} = 1 + 2\gamma_2$ (when $g_{12} = G_{12} = 0$).

For an isotropic surface w can be written as a function of two strain invariants. These are independent of the choice of parameters and, in the simple case considered here, take the form

$$I = \gamma_1 + \gamma_2, \quad J = \gamma_1 \gamma_2.$$

Thus

$$w = w(I, J). \quad (13)$$

The function should have a minimum for $\gamma_1 = \gamma_2 = 0$, and the simplest form is

$$w = A I^2 - B J \equiv \frac{1}{2} \frac{C}{1 + \mu} \left[\frac{1}{1 - \mu} (\gamma_1 + \gamma_2)^2 - 2\gamma_1 \gamma_2 \right] \quad (14)$$

with constant coefficients. In the last member, μ stands for Poisson's ratio, *i.e.* $\gamma_2 = -\mu\gamma_1$ when there is stress (12) in the u_1 -direction only (cf. eq. (2)). The expression is then reduced to $w = \frac{1}{2} C \gamma_1^2$, showing that C has the characteristic of a surface modulus of elasticity (it is the same constant that was introduced in (1)).

Eq. (14) can certainly be used in the case of infinitesimal strain when it corresponds to a linear stress-strain relation (Hooke's law); more generally it should be supplemented by higher-order terms:

$$w = A I^2 - B J + F I^3 - G IJ + \dots \quad (15)$$

Uniform expansion

The preceding formulas apply to any elastic membrane. We now turn definitively to the piece of parenchyma as depicted by the model. At zero turgor pressure, when the strip has a length l_0 , all cell-walls were assumed in (b) to be without strain. As the turgor pressure is allowed to rise, all cells are supposed to expand in a certain ratio without change of form (this is really a condition on the shape and arrangement of the cells connected with assumption (c) above). The piece as a whole will then expand in the same ratio (strip length = l). We write

$$\lambda = 1 + \varepsilon = l/l_0 \quad \text{or} \quad \varepsilon = (l - l_0)/l_0. \quad (16)$$

Then on any cell wall $G_{ik} = \lambda^2 g_{ik}$ and so

$$\gamma_1 = \gamma_2 = \frac{1}{2} I = \frac{1}{2} (\lambda^2 - 1) = \varepsilon + \frac{1}{2} \varepsilon^2, \quad J = \frac{1}{4} I^2 = \varepsilon^2 + \dots$$

It is now possible to relate the turgor pressure p to the state of stress of the cell walls. Suppose λ is varied by an infinitesimal amount $\delta\lambda$, the resulting variation of U , eq. (11), being

$$\delta U = \int \delta w \, dS_0 = \tau \frac{2S \delta\lambda}{\lambda} \quad (\tau = \delta S),$$

where $S = \lambda^2 S_0$ is the total area of all the distended cell walls in the strip and τ is the physical surface stress or tension ($= \tau_1 = \tau_2$, cf. eq. (12)), constant over the walls:

$$\tau = \frac{\partial w}{\partial I} + \frac{1}{2} I \frac{\partial w}{\partial J}. \quad (17)$$

On the other hand the work done by the excess internal pressure p in the same variation is

$$\delta W = p \delta V = 3p V \frac{\delta \lambda}{\lambda},$$

where $V = \lambda^3 V_0$ is the total volume of the cells (*i.e.* of the cell fluid). By the principle of virtual work δU and δW are to be equal under equilibrium conditions, so that

$$\tau = \frac{3p V}{2S} = \frac{pr}{2} = \lambda \frac{pr_0}{2} \quad (18)$$

with $r = \lambda r_0$ defined by

$$r = 3V/S. \quad (19)$$

Roughly speaking r is the "radius" of the distended cells and r_0 that of the undistended cells. In special cases the geometrical meaning can be made exact in a simple manner. According to assumption (c), V/S equals the ratio volume/area for any individual cell (the wall separating two contiguous cells is reckoned double), and if the cell form is that of a polyhedron with all its faces at the same distance from a common centre, then this distance is seen to equal r . In particular, for spherical cells r is indeed the cell radius.

For sufficiently small expansions we can use (14) and (17) to write the tension alternatively as

$$\tau = (4A - B) \frac{I}{2} = \frac{C}{1 - \mu} \varepsilon + \dots$$

Comparison with (18) shows that

$$\frac{p}{2} = \frac{D\varepsilon}{r_0} + \dots \equiv \frac{D}{r_0} \frac{l - l_0}{l_0} + \dots, \quad (20)$$

where $D = 4A - B = C/(1 - \mu)$. For future reference we also note the result

$$\frac{\lambda D}{r_0} \approx \frac{D}{r_0} + \frac{p}{2}, \quad (21)$$

which follows immediately from (20) with $\varepsilon = \lambda - 1$.

Stretching

On stretching the strip of parenchyma of turgor pressure p , the cell walls are further distorted, the cell volumes remaining invariant. We write Δl , $\Delta \gamma_{ik}$, etc., for the ensuing change in l , γ_{ik} , etc. In the model the cells are supposed so arranged, and the parameters so chosen, that $\Delta \gamma_{12} = 0$, and the change in the elastic potential w as given by (14) is then directly obtained in the form

$$\Delta w = \tau (\Delta \gamma_1 + \Delta \gamma_2) + A (\Delta \gamma_1 + \Delta \gamma_2)^2 - B \Delta \gamma_1 \Delta \gamma_2$$

with τ defined above. Here let us introduce

$$\bar{\gamma}_1 = \Delta \gamma_{11}/G_{11} = \Delta \gamma_1/\lambda^2, \quad \bar{\gamma}_2 = \Delta \gamma_{22}/G_{22} = \Delta \gamma_2/\lambda^2, \quad (22)$$

which play the same role in characterizing the deformations of the uniformly distended surface as the γ_i do for the undistended one, the invariants corresponding to I and J being $I = \bar{\gamma}_1 + \bar{\gamma}_2$, $J = \bar{\gamma}_1 \bar{\gamma}_2$. The total energy-change accompanying stretching can thus be written

$$\Delta U = \int \Delta w \, dS_0 = \tau \int I \, dS + \lambda^2 \int (AI^2 - B\bar{J}) \, dS, \quad (23)$$

where $dS = \lambda^2 dS_0$ is the distended surface element. As before the domain of integration is the total composite surface formed by all cell walls. The alternative form for the first term

$$\tau \int I \, dS = \tau \Delta S + \tau \int \left(\frac{1}{2} I^2 - 2\bar{J} \right) \, dS \quad (24)$$

(correct to second order in $\bar{\gamma}_1, \bar{\gamma}_2$) is sometimes useful; it is a consequence of the formula

$$\frac{\Delta dS}{dS} = (1 + 2I + 4\bar{J})^{1/2} - 1 = I - \frac{1}{2} I^2 + 2\bar{J} - \dots$$

In the derivation given here we started from the simplified expression (14) for w . A similar result follows from the general expression (13) or (15); it can be obtained from (23), if we make the substitution

$$\begin{aligned} A &\rightarrow \frac{1}{2} \left(\frac{\partial^2 w}{\partial I^2} + I \frac{\partial^2 w}{\partial I \partial J} + J \frac{\partial^2 w}{\partial J^2} \right) = A + (6F - G)\epsilon + \dots \\ B &\rightarrow - \frac{\partial w}{\partial J} = B + 2G\epsilon + \dots \end{aligned} \quad (25)$$

Our aim, already set down at the beginning, is to express ΔU as a function of the relative elongation $\alpha = \Delta l/l$ of the strip. The numerical relation will be slightly sensitive to details in the model; so it is hardly worth while to attempt a rigorous calculation for any particular variant of it. Instead, to get an idea of the possible range of variation of the results, within the limits of the model, we will evaluate (23) under two rather extreme assumptions about the behaviour of the cells on stretching. At first the structure as a whole is supposed to undergo a uniform deformation; afterwards the effect of allowing a greater freedom to the individual cell walls is studied in a special case.

Uniform deformation of the structure

For the present let us imagine the walls of all cells in the model to be completely grown together with those of contiguous cells. The following uniform deformation of the structure will then represent the average behaviour on stretching it. Any line element lying in the direction of the applied force is elongated in the constant ratio $(l + \Delta l)/l = 1 + \alpha$, while any line element perpendicular to the force is contracted in another constant ratio, determined by the requirement that all cell volumes should remain constant. To describe this deformation analytically, introduce a fixed coordinate system with origin at the centre of the strip and with z -axis in the direction of the force. If $\mathbf{r} = (x, y, z)$ is the position-vector before stretching of an arbitrary point on one of the cell walls, then

$$x \rightarrow \frac{x}{\sqrt{1+\alpha}}, y \rightarrow \frac{y}{\sqrt{1+\alpha}}, z \rightarrow z(1+\alpha). \quad (26)$$

(The argument does not mean that (26) must be the deformation actually realized in this form of the model, as local deviations may lead to a lower increase of the potential energy.)

We choose the parameter curves on a typical cell wall in such a way that the equation of the wall before stretching takes the form

$$x=x(u_1, u_2), y=y(u_1, u_2), z=z(u_1).$$

Writing $\partial x/\partial u_k = x_k$ etc., we have (cf. (10) and $ds^2 = d\mathbf{r} \cdot d\mathbf{r}$),

$$G_{ik} = \mathbf{r}_i \cdot \mathbf{r}_k = x_i x_k + y_i y_k + z_i z_k,$$

which on the assumed displacement (26) transforms into

$$G_{ik} + 2\Delta\gamma_{ik} = \frac{1}{1+\alpha} (x_i x_k + y_i y_k) + (1+\alpha)^2 z_i z_k = \frac{1}{1+\alpha} G_{ik} + 3\alpha z_i z_k, \quad (27)$$

apart from a term proportional to α^3 in the last member. Now $z_1 = \sqrt{G_{11}} \sin\psi$ and $z_2 = 0$, where ψ is the angle between the z -axis and the normal to the cell surface. Recalling the definitions (22), we obtain, to second order in α ,

$$2\bar{\gamma}_1 = \frac{1}{1+\alpha} - 1 + 3\alpha \sin^2\psi = \alpha(2 - 3\cos^2\psi) + \alpha^2, \quad 2\bar{\gamma}_2 = \frac{1}{1+\alpha} - 1 = -\alpha + \alpha^2.$$

Thus

$$I = \frac{\alpha}{2} (1 - 3\cos^2\psi) + \alpha^2, \quad J = -\frac{\alpha^2}{4} (2 - 3\cos^2\psi) \quad (28)$$

These expressions are to be inserted into (23). In the absence of external forces the equilibrium state should correspond to $\alpha=0$, indicating that there can be no term in ΔU proportional to α . Consequently,

$$\int (1 - 3\cos^2\psi) dS = 0 \quad (29)$$

(which is equivalent to saying that the total area of the cell-walls in our model is a minimum for $\alpha=0$). With the help of (28) and (29), ΔU reduces to an expression which can be written

$$\Delta U = \frac{1}{2} \left[\frac{2\tau S}{V} + \frac{\lambda^2 S}{2V} (B-A) + \frac{9\lambda^2 A}{2V} \int \cos^4\psi dS \right] V \left(\frac{\Delta l}{l} \right)^2.$$

If the total volume V of the cells is equated to the volume V of the parenchyma strip — which means neglecting the volume of the intercellularies — the formula for Young's modulus of the cell-structure follows immediately on comparison with (9):

$$E = 3p + \frac{3}{2} \left(\frac{p}{2} + \frac{D}{r_0} \right) \left(\frac{B-A}{D} + \frac{9A}{D} \cdot \frac{1}{S} \int \cos^4\psi dS \right). \quad (30)$$

We have here introduced p through (18) and used (19) to write $\lambda^2 S/V = 3\lambda/r_0$. Finally, λ/r_0 has been rewritten with the help of (21), which means that higher

powers of p than the first are neglected. Note that the right-hand side of (30) should properly be multiplied by V/V .

Only the integral in the last term is model-dependent. However, its *average value* for all possible directions of the force with respect to the cell-structure is quite independent of the cell-form: the factor $\cos^4\psi$, averaged over all orientations in space, is seen to equal $1/5$. Since $2A/D=1/(1+\mu)$, $B/D=(1-\mu)/(1+\mu)$ (cf. (14), and $D=C/(1-\mu)$), we get in this way

$$E=3p+\left(\frac{p}{2}+\frac{D}{r_0}\right)\frac{3(7-5\mu)}{10(1+\mu)}. \quad (31)$$

It should be noted that this expression corresponds to the approximate form (14) for w . More generally we have to make the substitution (25) in (30), leading to

$$E=\left[1+\frac{7-5\mu}{20(1+\mu)}+\frac{3(4F+G)}{10D}\right]3p+\frac{3(7-5\mu)}{10(1+\mu)}\cdot\frac{D}{r_0} \quad (32)$$

in first order. (For simplicity F and G are again put equal to zero in the following formulas.)

In a regular cell-structure the E -value calculated from (30) will vary about the average (31) for different directions of the force. To illustrate the variation a few examples are added (incidentally, the correctness of (29) is easily verified for the individual cells in these examples). Except in the first instance, the cells are taken to be polyhedra filling the space compactly.

Spheres (radius= r). A strictly spherical model, e.g. that of Figure 1, does not fall under the assumptions made in this section. However, it may be pointed out for comparison that the result of evaluating (30) for such a model would again be (31). But in this case the factor V/V suppressed above would be important, its maximum value being $\pi\sqrt{2}/6=0.740$ (when each sphere touches twelve others — densest packing); for the cubic packing of Figure 1 the ratio is $\pi/6=0.524$.

Rhombic dodecahedra (edge= $r\sqrt{3}/2$). This polyhedral form, bounded by twelve rhombic faces (Figure 3 B), can result if spheres in a state of densest packing are allowed to expand so as to fill up the cavities. We take the orientation of the cells to be such that one of the three axes is in the z -direction ($\cos^2\psi=1/2$ on eight faces and 0 on the rest). Then

$$E=3p+\left(\frac{p}{2}+\frac{D}{r_0}\right)\frac{3(5-4\mu)}{8(1+\mu)}.$$

Isodiametric tetrakaidecahedra (edge= $r(1+2\sqrt{3})/4\sqrt{2}$). This polyhedron, with six square and eight hexagonal faces, can be pictured as a regular octahedron with all six corners truncated (Figure 3 A). For all cells oriented with two opposite squares and four hexagons parallel to the z -axis ($\cos^2\psi=0$ on these faces, $1/2$ on the remaining squares, and $2/3$ on the remaining hexagons), we get

$$E=3p+\left(\frac{p}{2}+\frac{D}{r_0}\right)\frac{3(67-2\sqrt{3}-44\mu)}{88(1+\mu)}. \quad (33\text{ a})$$

For two square faces perpendicular to the z -axis ($\cos^2\psi=1$ on these squares, 0 on the remaining ones, and $1/3$ on all hexagons):

$$E=3p+\left(\frac{p}{2}+\frac{D}{r_0}\right)\frac{3(10+2\sqrt{3}-11\mu)}{22(1+\mu)}. \quad (33\text{ b})$$

Cubes (edge = $2r$). If two faces are perpendicular to the z -axis ($\cos^2\psi = 1$ on these two faces and 0 on the other four), we get

$$E = 3p + \left(\frac{p}{2} + \frac{D}{r_0} \right) \frac{3(2-\mu)}{2(1+\mu)},$$

and if one diagonal lies in the z -direction ($\cos^2\psi = 1/3$ on all faces):

$$E = 3p + \left(\frac{p}{2} + \frac{D}{r_0} \right) \frac{3(1-\mu)}{2(1+\mu)}.$$

Freely moving cell walls — spherical model

It was assumed in the calculation above that the structure as a whole undergoes a certain uniform deformation. To judge the effect of relaxing this restriction, let us make a formal calculation in which the walls are allowed to move independently of each other (of course this is an extreme case without any histological foundation). We shall only consider the case of spherical cells, again deformed to ellipsoidal shape on stretching, but now without any restrictions being imposed on the displacement along the surface. The transformation (26) is then to be combined with such a displacement, which, for ease of visualization, we may always describe as taking place first. It is seen to be of the form

$$\theta \rightarrow \vartheta(\theta),$$

where θ is the azimuthal angle on the sphere before, and ϑ after, this first internal distortion.

The original squared line element on the sphere is of the form

$$ds^2 = r^2 d\theta^2 + r^2 \sin^2 \theta d\varphi^2$$

in polar coordinates θ and φ , used as surface parameters u_1, u_2 . According to the above assumptions it is transformed into

$$ds^2 \rightarrow \left(\frac{1}{1+\alpha} + 3\alpha \sin^2 \vartheta \right) r^2 d\vartheta^2 + \frac{1}{1+\alpha} r^2 \sin^2 \vartheta d\varphi^2 \quad (34)$$

on the ellipsoid as a result of the stretching (cf. (27)). We write

$$\vartheta = \theta + \alpha h \sin \theta \cos \theta \quad (35)$$

to bring out the fact that $\vartheta = \theta$ for $\theta = 0, \pm \pi/2$, as follows from considerations of symmetry. It will be convenient to regard the factor h as a function of $\zeta = \cos \theta$, so that $dh = -h' \sin \theta d\theta$. On introducing ϑ from (35) into (34), we can obtain the strain components $\bar{\gamma}_1, \bar{\gamma}_2$ along the same lines as before. The result is

$$\begin{aligned} \bar{\gamma}_1 &= \left[\frac{1}{2}(2 - 3\zeta^2) - h(1 - 2\zeta^2) - h'\zeta(1 - \zeta^2) \right] \alpha \\ \bar{\gamma}_2 &= \left[-\frac{1}{2} + h\zeta^2 \right] \alpha, \end{aligned}$$

if we only retain first-order terms in α . As follows from (23) and (24), this is all that is needed here because I^2 and \bar{J} are then given correctly in second order and the

term with ΔS , being proportional to α^2 and independent of h , is more directly evaluated for $h=0$. In fact, the sum of all terms independent of h can be taken immediately from (31).

In this way one obtains, after some partial integrations and using (19),

$$\Delta U = \frac{V\alpha^2}{2} \left[E + \frac{3}{2r} \int_{-1}^1 d\xi \xi^2 (1-\xi^2) \{ (12H-K)h^2 - 3(4H+K)h + 2Hh'^2(1-\xi^2) \} \right], \quad (36)$$

where \bar{E} is the value given by (31), and

$$H = \frac{1}{2}\tau + \lambda^2 A, \quad K = 2\tau + \lambda^2 B.$$

The surface distribution characterized by h tends to be such as to make ΔU as small as possible. In the absence of any restrictions on h we have an ordinary variational problem to be handled by standard methods. As is easily established, it is solved by a constant function h , viz.

$$h = \frac{3(4H+K)}{2(12H-K)}. \quad (37)$$

The new value of Young's modulus is obtained on inserting \bar{E} and h into (36). For $\tau \ll D$, i.e. $\varepsilon \ll 1$, the result can be written approximately

$$E = \frac{6}{5} \left[1 + 6 \left(\frac{1+\mu}{5+\mu} \right)^2 \right] p + \left(\frac{p}{2} + \frac{D}{r_0} \right) \frac{12(1-\mu)}{5(5+\mu)}. \quad (38)$$

A factor V/V is to be understood in the right member.

Discussion

In applying the results obtained above to potato tuber parenchyma, we note that the volume taken up by the intercellular spaces is in reality quite negligible and that the cell walls are thin. The approximation $V/V \approx 1$ adopted in the formulas should therefore be a good one. Further, the parenchyma is on the whole isotropic, the individual cells being irregular and oriented at random. It is true that we have only treated certain regular structures, but granted the general assumptions of the model the averaged value of Young's modulus obtained in (31) is independent of the particular cell-form. So we accept (31) or rather (32), as our best general approximation for an isotropic cell-structure in which no individual motion of the cell walls is possible.

Here some ambiguity is due to the fact that constants like F and G in (32) cannot be determined in the experimental procedure mentioned above. Even though it would be overrating the isotropic membrane approximation to insist on the detailed form (15) for w , leading to (32), the result does show that the exact dependence of E on turgor pressure can only be decided on the basis of a rather detailed knowledge of the elastic properties of the cell-wall material. The choice $F=G=0$ corresponding to (31) is more or less arbitrary.

A further uncertainty is connected with the considerations in the last section, where the uniform deformation of the structure assumed in (26) was allowed to have an additional internal distortion of the individual cell walls superimposed upon it.

The extra freedom postulated to get (38) is certainly an exaggeration, but some reduction of the coefficients in (31) is to be expected. As in (37), let us put $h=c$ (constant), and then let us see how the value of E yielded by (36) varies with c . If the expression is written in the form

$$E = ap + b \frac{D}{r_0}, \quad (39)$$

and if, for definiteness, μ is chosen to be $1/3$ (which is reasonable), one obtains

$$a = \frac{18}{5} \left[1 - c \left(1 - \frac{4c}{9} \right) \right], \quad b = \frac{6}{5} \left[1 - 2c \left(1 - \frac{2c}{3} \right) \right].$$

Here $c=0$ corresponds to (31): $a=18/5$, $b=6/5$. Evidently, for small c -values b is relatively more sensitive to changes in c than a is. Thus for $c=1/4$: $a=14/5$, $b=7/10$, while $c=3/4$ leads to (38): $a=9/5$, $b=3/10$.

To sum up: even if the general assumptions of our model are accepted, there is still room for speculation about the exact elastic properties of the cell walls and the degree of adherence between the walls. This causes some uncertainty in the theoretical predictions. For not too high values of p , Young's modulus E should be expressible in the form (39), and the numerical coefficients are expected to be approximately $a \approx 3$ and $b \approx \frac{1}{2}$ to 1, but we can only feel confidence in their order of magnitude.

Summary

The rigidity (or rather Young's modulus) of potato tuber parenchyma and its dependence on turgor pressure is investigated theoretically on the basis of a simple model. In the model the cells of the parenchyma are approximated by more regular geometrical cell-forms (spheres or polyhedra), each cell being bounded by an elastic membrane (cell wall) and filled with an incompressible fluid (cell sap). It is shown that this model yields the correct dependence of cell diameter on turgor pressure and that certain cell-wall constants can be determined using this relation.

If a stress is applied to this model of the parenchyma, each cell is elongated in the direction of the stress, its volume remaining constant because of the incompressible cell-fluid. In such a deformation the area of the cell surface will increase, which is possible by a stretching of the elastic cell walls. According to the model, this is the general mechanism behind the elasticity and rigidity of the tissue. A mathematical theory elaborating this conception yields results for Young's modulus E and its dependence on turgor pressure that are in good agreement with the experimental values, both qualitatively and quantitatively. Certain apparent deviations of the behaviour of the parenchyma from Hooke's law are briefly discussed.

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The Effect of Gibberellins and Indole-3-Acetic Acid on the Root Cells of *Narcissus Tazetta* (L.)

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Introduction

It has been established by numerous investigators that the gibberellins considerably increase the growth of plants, especially of dwarf forms. They increase the area of the leaves and the fresh and dry weight of the plants. In many plants they improve the flowering and the germination of the seeds (Wittwer and Bukovac 1957).

The actual mechanism of growth stimulation, *i.e.*, how the cells react to the gibberellins, has attracted much less attention. A few papers on the cytological effects of gibberellins have been published, however, (Y. Kato 1955, Berger 1957). In these studies the experimental plant has been the onion. The aim of the present study has been to investigate the effect of gibberellins on the root cells of *Narcissus tazetta* and to compare the results with the effect of indole-3-acetic acid on the roots of the same plants.

Material and Methods

Narcissus tazetta (commercial name Totus albus) was used as the experimental plant. It is a suitable plant for such experiments, since its bulbs produce a great number of roots. For the sake of comparison, a few experiments were made using the roots of the onion, *Allium cepa* (L.).

The bulbs were placed on glass jars (350 ml.) filled with tap water. The exper-

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iments were carried out at room temperature (20—21°C). When the roots had reached a length of 5—10 cm. the jars were filled with the solutions to be tested.

Both the potassium salt of the gibberellic acid (Gibrel, Merck) and the gibberellic acid itself (Gibberellic acid, Plant Protection LTD) were used. The concentration used varied from 20 to 200 mg. per 1000 ml. distilled water (=20—200 p.p.m.). The time of treatment of the roots varied from 4 hrs. to 11 days. Some of the roots were made into preparations immediately after the treatment. Some of the bulbs were transferred from the gibberellic solution to tap water for a few hours to recover, and roots were fixed after this.

In another series of experiments the roots of *Narcissus tazetta* were treated with a solution of 5 p.p.m. indole-3-acetic acid. The times of treatment were 1, 2, 4, 8, and 10 days, after which the roots were fixed.

Preparations were made both of the root tip cells and the differentiated cells some 1—2 cm. from the root tip. Most of the material was made into squash preparations, being fixed in acetic-alcohol (1 : 3). Some of the roots were fixed in Craf-solution, embedded in paraffin and sectioned either transversely or longitudinally. All the preparations were stained according to the Feulgen technique (Darlington and La Cour 1947).

Observations

The concentration of the gibberellin solutions varied from 20 p.p.m. to 200 p.p.m. No abnormalities in root development could be detected with any of the treatments. When the roots were transferred from the gibberellin solutions to tap water, they continued growth undisturbed.

The root tip cells divided in all the gibberellin concentrations used (Figure 1). No significant variation in the frequency of mitoses could be observed between the different gibberellin solutions or as compared with the untreated root tips. Neither did the time of treatment cause noticeable changes in the mitotic frequency. However, a period of treatment as long as 11 days in 100 p.p.m. solution seemed to prevent cell division in the root tips.

The cells situated in the differentiating region of the root are, as a rule, in the resting stage. Under certain experimental circumstances the gibberellins induce mitoses in them (Figures 2—3). During the period September—November the gibberellin concentrations used, *viz.* 20—200 p.p.m., did not induce the differentiated cells to divide, apart from an occasional solitary mitosis. The experiments carried out during December—February, however, gave different results. The differentiated cells of the onion roots did not divide, but the preparations made of the roots of *Narcissus tazetta* revealed a great number of mitoses. The gibberellin concentration used was 70 p.p.m. The cells of the root cortex seemed to divide most frequently (Figure 3), but various mitotic stages could also be observed in the vascular bundle cells, which have long, narrow nuclei (Figure 2) and in the large pith cells. The longitudinal

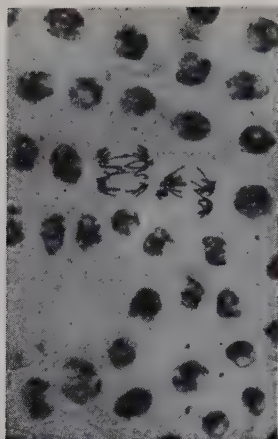


Fig. 1.

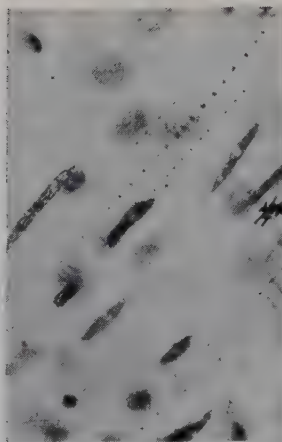


Fig. 2.

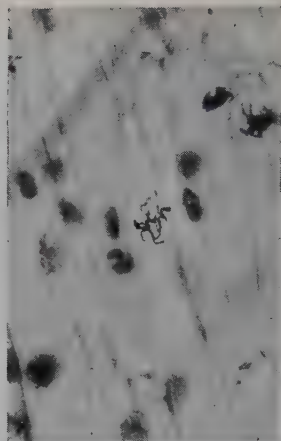


Fig. 3.

Figure 1. *Dividing root tip cells of Narcissus tazetta* (4 hrs. in 100 p.p.m. gibberellin).

Figure 2. *Dividing differentiated root cells in the vascular bundle of Narcissus tazetta* (1 day in 70 p.p.m. gibberellin).

Figure 3. *Dividing differentiated cells in the root cortex of Narcissus tazetta* (1 day in 70 p.p.m. gibberellin).

sections showed that the mitoses were not distributed evenly throughout the root, but were concentrated in certain regions.

The experiments showed that the gibberellins did not have any effect on the course of mitosis. The division of both the root tip cells and the differentiated cells was quite normal. No disturbances were visible in the anaphases or in the individual chromosomes (Figure 1). No polyploid cells could be observed.

The experiments with indole-3-acetic acid were carried out in September and the concentration used was 5 p.p.m. This treatment induced the differentiated cells to divide. Mitoses were most frequent after 1—2 days. Divisions were seen in all cell types, except the epidermal cells. The course of mitosis was normal and no structural changes could be observed in the chromosomes.

Discussion

Plants are able to stand a considerably wider range of gibberellin concentrations than of auxins. The gibberellins promote plant growth evenly and do not give rise to such disturbances as do the auxins. Neither do they prevent root growth as do the auxins.

The growth of plants is caused by either cell division or cell enlargement or both. It has been found that when treated with auxins some tissues react more readily than others. Auxins promote the enlargement of certain cells, *e.g.* the epidermal and cortical cells. They also promote the division of cambial cells and thus the formation of secondary xylem and phloem. Auxin treatment intensifies the phellogen activity and in certain regions the cortex and pith cells also begin to divide. Separate groups of dividing cells are thus formed. Often several rings of vascular bundles are formed, which confers a wavy structure on the stem (Palser 1942—43).

In the earlier studies concerning the effect of gibberellins on the cells, attention was first paid to the enlargement of the cells and the gibberellins were claimed to have the same effect as the auxins. Kurosawa (1928) found that gibberellins cause a lengthening of the epidermal and parenchymatous cells in rice (*cf.* Stowe and Yamaki 1957). In other plants, too, growth of cells but not their division was observed. According to Y. Kato (1955), the lengthening of the internodes by gibberellins was caused by the growth of the cells.

It is probable, however, that the cells must be induced to divide, for their enlargement alone is not a sufficient explanation for the enormous increase in growth caused by the gibberellins. According to J. Kato (1958), gibberellins do not induce cells to divide in cut pieces of stem nor do they give rise to callus formation. Bradley and Crane (1957) have found that gibberellins promote cambial activity in apricot stems. This is seen in the increased xylem formation in apricot spur shoots.

The effect of gibberellins on the root cells of the onion has been studied by Y. Kato (1955) and Berger (1957). According to Y. Kato, a 100 p.p.m. solution considerably retards the division of the root tip cells. After 2 days all the nuclei are in interphase. In the present experiments, however, this concentration was not found to be damaging to the roots of *Narcissus tazetta*, and a 70 p.p.m. solution did not prevent mitoses in the onion roots. Numerous dividing cells were visible in the root tips. After treatment for 11 days, however, practically no mitoses were seen in the roots of *Narcissus tazetta*. According to Y. Kato, a solution as weak as 10 p.p.m. somewhat inhibits the growth of the roots. The results obtained in the present experiments are not in agreement with these.

The gibberellin treatment induced differentiated cells to divide in the roots of *Narcissus tazetta*. The cells of the cortex were the most frequent to divide, but the narrow cells of the vascular bundles and the pith cells also showed mitoses. The divisions were not evenly distributed throughout the roots but were concentrated in certain regions. According to Y. Kato (1955), the differentiated cells of the onion were induced to divide with gibberellins and the divisions were localized in certain areas in the large pith cells. In Berger's

(1957) experiments, gibberellins did not induce differentiated cells to divide in onion roots. Instead he stresses that the divisions are accelerated in the the root tips.

In the present experiments, on the contrary, differentiated cells divided in response to the gibberellin treatment. It may be remembered, however, that earlier in the autumn the differentiated cells did not divide, mitoses being observed in them only during the winter months December—February. It seems that plants are more responsive in the later part of their rest period. Therefore the time of year may influence the results obtained. A possible explanation of the conflicting observations made on the onion might be differences in the time of year at which the experiments were carried out, though this has not been mentioned by the authors.

The effect of growth substances on the roots of various plants has been intensively studied (cf. D'Amato 1952). With this treatment the external appearance of the roots is simultaneously changed. A tumour is formed above the growing point; this is mainly due to enlargement of the cells. At the same time, the root tip cells cease to divide, even when exposed to very low concentrations. Instead, the differentiated cells above the growing point being to divide (Therman 1951, Y. Kato 1955). Likewise in the present experiments, the treatment with indole-3-acetic acid induced the differentiated cells to divide. Mitoses were observed in cells of all types except the epidermis.

The gibberellins do not seem to affect the course of mitosis neither do they cause any changes in the chromosomes. All the mitotic stages observed were regular, at least in the roots of *Narcissus tazetta* and in the onion roots. Y. Kato (1955) has claimed that the gibberellins give rise to changes in the chromosomes of the onion roots and especially to disturbances during anaphase. The primary effect is that the chromosomes become sticky. During continued treatment pseudochiasmata are formed which disturb the course of the anaphase and telophase. No such phenomena could be observed in the present experiments. Berger (1957) also disagrees with Y. Kato, having observed no pseudochiasmata. However, Berger found that a 100 p.p.m. solution affected the appearance of the chromosomes in the roots of the onion. These became shorter and attained a segmented appearance. The daughter chromosomes in prophase lay clearly apart from each other and were abnormally spirialized. No such phenomena were observed by the present author in the roots of *Narcissus tazetta*.

All the divisions in the roots of *Narcissus tazetta* were normal and diploid. The gibberellins do not cause polyploidy. According to Y. Kato (1955), the gibberellins give rise to polyploid cells in the roots of the onion. Berger, however, has refuted this claim, and the present observations corroborate Berger's opinion.

Neither did the treatment with indole-3-acetic acid cause any disturbances in the mitoses or the individual chromosomes in the roots of *Narcissus tazetta*. No polyploid cells were observed. Probably the gibberellins and indole-3-acetic acid only reveal previously existing polyploidy in the onion roots by compelling the cells to divide (cf. D'Amato 1952). The present observations show that the cells of the roots of *Narcissus tazetta* are diploid.

Brian and Hemming (1955) were of that opinion that the inclusion of the gibberellins in the auxins can be cytologically justified by the fact that both groups of substances lead to the enlargement of cells. The present observations show that they also have similar effects on cell division in the roots of *Narcissus tazetta*. Both promote the division of differentiated cells and do not cause any disturbances in the course of mitosis or the structure of the chromosomes. In contrast to the auxins, the gibberellins do not prevent the division of root tip cells, even in high concentrations. The strong growth-promoting effect of the gibberellins is obviously due to the fact that they induce differentiated cells to divide and yet do not prevent cell divisions at the growing points.

Summary

1. The gibberellins do not prevent the division of the root tip cells of *Narcissus tazetta* in concentrations between 20—200 p.p.m. during a 4-hr. to 10-day period of treatment.

2. The gibberellins induce the differentiated cells to divide in the roots of *Narcissus tazetta*.

3. The gibberellins do not affect the course of mitosis.

4. The gibberellins give rise neither to changes in the chromosomes nor to polyploidy.

5. Indole-3-acetic acid also compels the differentiated cells in the roots of *Narcissus tazetta* to divide. A longer treatment, however, prevents the division of the root tip cells.

6. Indole-3-acetic acid gives rise to the enlargement of the cortical cells above the root tip. The gibberellins do not have this effect.

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A Study of Sirenin, the Chemotactic Sexual Hormone from the Watermold *Allomyces*

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One step in the sexual reproduction of the watermolds in the subgenus *Allomyces* is the fusion of a small, orange, motile, male gamete with a larger, colorless, motile, female gamete. The female gametes release into the surrounding aqueous medium a substance, recently named "sirenin" (1), which attracts the male gametes (2). This sexual hormone passes through a dialyzing membrane, is soluble in fat solvents, and is stable to light and to heating for one hour at 100°C. if in neutral solution but not in acidic or basic solutions.

A small, highly active sample of sirenin has been prepared. The method of purification and the results of elementary, ultra-violet, infra-red, and mass spectrographic analyses are presented in this paper.

Materials and Methods

The organisms used were the M-4 male strain and the F-2 female strain derived, as previously described (2), from a cross between *Allomyces macrogynus* and *A. arbuscula*. Stock cultures were carried on nutrient agar slants with mycelial transfers as needed. The nutrient agar for both slants and plates contained 1.0 g. K_2HPO_4 , 0.5 g. $MgSO_4 \cdot 7H_2O$, 15 g. soluble starch, 4 g. yeast extract, and 1000 ml. double distilled water (3). The DS solution referred to below contained 0.0005 M KH_2PO_4 , K_2HPO_4 , and $(NH_4)_2HPO_4$ and 0.00005 M $MgCl_2$ and $CaCl_2$.

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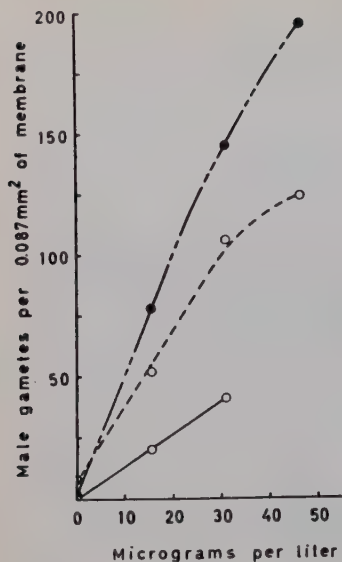


Figure 1. Representative standard curves for the bioassay of sirenin.

The bioassay was performed as previously described with minor modifications (2). These were: (a) washing the male plants in one large (ca. 500 ml.) volume of water for an hour instead of four successive quarter hour changes of water; (b) continued re-use of the membrane in the bioassay apparatus provided the apparatus was kept in water between uses and that both surfaces of the membrane were swabbed with wet cotton before each use; (c) use of the same suspension of male gametes for two to three successive assays.

Of more importance was the quantitative use of the bioassay. Previous difficulties were found to be related to the physiological condition of the male gametes as well as their concentration. When both these factors were optimum, then standard curves such as those illustrated in Figure 1 were obtained. Quantitative results obtained at one time were quite reliable; on the other hand, results obtained with different suspensions of male gametes can differ by as much as 30 per cent.

Early in the investigation an unpurified chloroform solution of sirenin was prepared such that 10 μ l. dissolved in 1.0 ml. H_2O (after evaporation of the chloroform) gave a detectable male gamete count per 0.087 mm.² of the membrane. With most suspensions of male gametes a linear response was obtained up to about 100 μ l. of the chloroform solution per 1.0 ml. of water. Higher concentrations almost always failed to increase the readings substantially. One unit of activity was made equal to 1 μ l. of chloroform solution per ml. of water. This standard was used throughout the investigation. When, as described later, a presumably pure preparation of sirenin was obtained, the standard was calibrated against a solution of this preparation. One μ l. of the standard was found to contain 0.62 μ g. of sirenin (assuming the sample to be pure sirenin). Most of the quantitative data presented in this report has been transformed from the arbitrary units to actual weights using the preceding conversion factor.

The area of membrane over which male gametes were counted was 0.087 mm.². The magnification used was 160. In the previous paper (2) the area counted was given as 0.0005 mm.². This was an error and should have been 0.05 mm.².

Results

Production

Female plants were grown in 500 ml. of medium contained in 1800 ml. Fernbach flasks. These flasks were processed in sets of nine, the number accommodated by a shelf on the shakers. The medium was the same as that used for the nutrient agar plates (less the agar).

Two types of inoculum were used. The first was prepared from female plants grown on agar plates. The second was prepared from the plants resulting from the first type of inoculum. Further successive use of plants grown in liquid medium was avoided because of the possibility that the cultures would gradually become sporophytic as a result of the parthenocarpic development of female gametes and of the presence of some zygotes which would also develop into sporophytic plants.

For the initial inoculum, medium sized plates (about 70 mm. diameter) were inoculated at five points with mycelial transfers of female plants. After incubating a week at 25°C. the plates were just covered with the fungus. The entire contents of two plates plus 100 ml. of sterile, distilled water was fragmented in a blender for 10 to 20 seconds at high speed. Approximately 10 ml. of the suspension was then added to each flask. The secondary inoculum was prepared by pouring into the blender cup the plants produced in one flask plus about 100 ml. of the medium. After fragmentation, approximately 10 ml. of the suspension was added to a flask. The flasks were then incubated for three days at 25°C. or two days at 30°C. on shakers. Space was available for 18 flasks at 30°C. and 9 at 25°C. The primary inoculum developed into discrete balls whereas the growth from the secondary inoculum approximated a mush.

After growth, the plants were strained through a cloth hand towel and then distributed into 22 cm. diameter glazed clay plates each containing about 150 ml. double distilled water. Under these conditions female gametes were produced and discharged, the process continuing for about 48 hours provided the water was replaced at intervals. The normal schedule involved moving the plants into fresh water after 3, 8, and 24 hours. Each set of nine flasks thus yielded about two liters of water containing sirenin, other substances, and gametes. These solutions were first filtered with suction through two

Table 1. *Concentration of hormone in representative initial aqueous solutions.*¹

Solution No.	Volume ml.	Concentration μ g. pr l.
1	980	73
2	680	85
3	330	89
4	820	66
5	920	29
6	1070	104
7	925	29
8	1410	28
9	1150	111
10	1100	68
11	1100	64
12	1000	87
No. 11 after absorption by charcoal		3
No. 12 after absorption by charcoal		3

¹ The 12 solutions represent the total collections from approximately 60 flasks of plants.

layers of No. 3 filter paper and then through Seitz filters to remove the gametes and plant debris. At this stage the sirenin concentration averaged about 70 μ g. per liter and ranged from 25 to 110 μ g. per liter (Table 1).

Concentration

Initially the only method available for processing the daily accumulation of crude, aqueous solution was boiling under vacuum to a small volume and then extracting the lipoidal material with chloroform. The chloroform was readily evaporated at room temperature by bubbling air vigorously through the chloroform solution contained in a wash bottle. There were indications, however, that as much as 50 per cent of the sirenin was lost during the boiling. The problem was solved when it was found that charcoal effectively removed the sirenin (Table 1) together with much other material, from the solutions. After a few crude tests the standard practice was to add a spoonful (approximately 5 g.) of Norit FNX Special charcoal to each 500 ml. of solution. The charcoal was then dried at room temperature and stored until 50 to 100 g. had accumulated.

The accumulated charcoal was mixed with twice its volume of chloroform to elute the sirenin. After standing at least two hours with occasional agitation, the chloroform was drained off with suction. The elution was repeated twice more and the chloroform then evaporated to dryness at room temperature leaving a dark-yellow, fatty material.

Table 2. *Recovery of hormonal activity in effluent from silicic acid column.*¹

Fraction No.	Volume ml.	Activity ² units
1	49	0 ³
2	10	1,600
3	10	0 ³
4	10	1,000
5	10	80,000
6	10	216,000
7	10	192,000
8	10	192,000
9	10	76,000
10	10	52,000
11	10	13,200
12	10	99,200
13	10	25,200
Alcohol precipitate removed from original sample		9,100
Total activity recovered		858,500
Activity in original sample ³		950,000

¹ The influents and effluents of this run of the column were the same as those illustrated in Fig. 2.

² As later ascertained, a unit of activity results from 0.62 µg. hormone per liter.

³ On the basis of analyses of other fractionations these fractions probably contained a small amount of hormone judged to be about 10,000 units for fraction 1 and 1,000 for fraction 2.

Purification

The first substantial progress towards purification of this residue was made when absorption chromatography on silicic acid-impregnated paper (4) separated the hormonal activity from impurities detectable by ultra-violet light and iodine staining followed by development with starch (4). The R_f of the sirenin was 0.75. This success, after failure with more than thirty other paper chromatographic procedures used for the separation of various kinds of lipids, led directly to the use of a silicic acid column.

To anticipate subsequent results, it was found that the chloroform residue could not be used directly in the column. There were apparently sufficient nonadsorbed impurities in the crude extract to carry the sirenin through with them. The chloroform residue was therefore reextracted with water to effect a substantial purification. In practice, the residue was taken to dryness at room temperature on the bottom of a flat 6×8 cm. rectangular, glass container. From 5 to 20 ml. of redistilled water was added for periods of 15 to 120 minutes over a total span of 6 to 8 hours. If, upon assay of the residue, any substantial amount of activity was found, the extraction was repeated. The aqueous solution was filtered through No. 00 paper into a 20 by 30 cm. baking dish and the water evaporated at approximately 30°C. on the hot

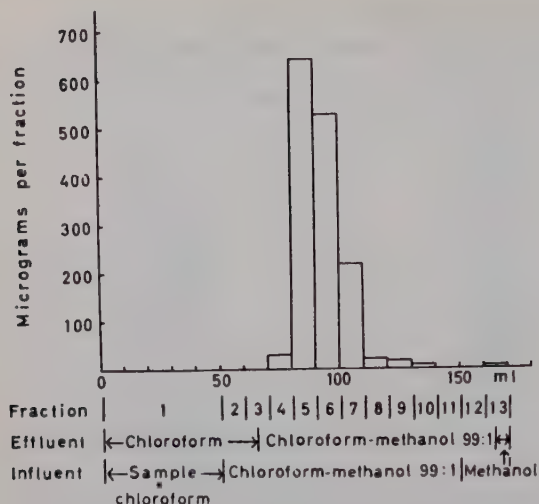


Figure 2. Fractionation of sirenin on a silicic acid column.

water radiator. The dish was then washed with small quantities of redistilled chloroform. After the chloroform was evaporated down to a volume of 3 to 10 ml. it was ready for fractionation on the column.

A column 1.1 cm. in diameter, 20 cm. long, and with a hold-up volume of 15 ml. was prepared with 10 g. of 100 to 200 mesh silicic acid (5). Preliminary trial runs with different concentrations of methanol in chloroform showed that chloroform-methanol 99:1 was a satisfactory eluant. The standard sequence of influents thereafter was: (a) 3 to 5 ml. sample followed by chloroform for a total volume of 50 ml.; (b) 100 ml. of chloroform-methanol 99:1; (c) 20 ml. methanol; and (d) 50 ml. of chloroform to leave column ready to receive another sample. The effluent upon adding (a) was collected as a single fraction and usually discarded. The effluent upon adding (b) and (c) was collected in 10 ml. fractions. The bulk of the impurities, at least the colored ones, were eluted by the methanol. Usually the effluent upon adding (c) was also discarded unless it contained considerable activity. If so, it was refractionated. The recovery from the column was almost complete as shown by Table 2.

When this procedure was finally perfected all the available material in the laboratory in the form of several separate samples, was passed through the column as described. Fractions 1, 12, and 13 from each run were discarded. Fractions 2—11 of the several runs were combined, reduced in volume, and the resulting sample then again fractionated. This fractionation is described in Figure 2. Fractions 5 and 6 were combined and yielded, upon evaporating off the solvent, 1.93 mg. of material. The assays of this sample are shown in

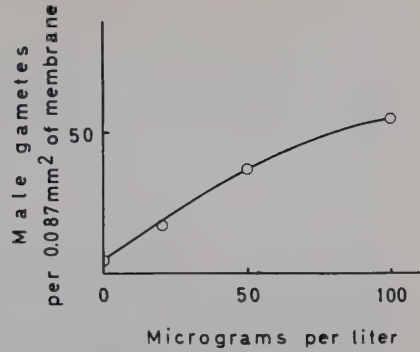


Figure 3. *The biological activity of low concentrations of a purified preparation of sirenin.*

Figures 3 and 4. It was unexpected to find that this highly purified material evoked an almost linear response to concentrations equal to approximately 1600 arbitrary units of activity whereas a linear response up to only about 100 units characterized the standard unpurified solution of sirenin. Under the best assay conditions a detectable response was obtained with 10 $\mu\text{g.}$ per liter. The material obtained from fractions 5 and 6 was used to calibrate the standard solution and for the analyses reported below.

One of the many unsuccessful attempts at purification should be mentioned here because of the light it sheds on the chemical nature of sirenin. The low solubility of sirenin in water as indicated by the slowness of the water extrac-

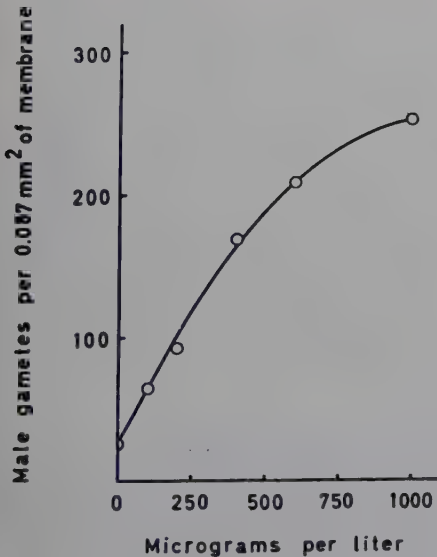


Figure 4. *The biological activity of high concentrations of sirenin.*

tion described above and its ready solubility in fat solvents suggested a neutral molecule. This was more explicitly demonstrated when it was found that the sirenin was not retained upon passage (in a 50 per cent alcohol solution) through either Dowex 50 or Dowex 2 ion exchange columns.

Analyses

The inferences to be drawn from the analyses presented here are strictly tentative since they are based on a single sample whose purity was unknown and can be appraised only on the basis of the purification process and the biological activity of the preparation. The data are reported for whatever aid they may provide for the ultimate determination of the structure of sirenin.

General considerations. — The solubility properties of sirenin and its failure to be adsorbed by either cation or anion ion exchange resins indicate a neutral molecule. The molecule would appear not to be unduly large since it passes through a dialyzing membrane; this diffusion is relatively fast since a reaction can be observed in the bioassay in as little as five minutes when the test solution is of reasonably high concentration.

Ultra-violet spectrum. — The ultra-violet spectrum yielded no positive information in that it showed only the absorption at the lower wave lengths characteristic of organic compounds lacking specific ultra-violet absorption properties. The lack of characteristic absorption bands excludes a number of chemical groupings from consideration.

Infra-red spectrum. — The information derived from the infra-red spectrum is the most reliable of that presented here since small amounts of impurities are not likely to interfere with the spectrum of the major component. There was a broad, not very well resolved absorption band consisting of the sum of several overlapping bands in the $6\ \mu$ double bond region. A peak at $5.63\ \mu$ indicated with reasonable certainty a lactone ring, a marked peak at $6.85\ \mu$ pointed to ketone and/or aldehyde functions, and several perturbances in the 5.95 to $6.03\ \mu$ region suggested double bond carbon-carbon or carbon-nitrogen functions. The spectrum gave no evidence of aromatic rings and showed no positive evidence for hydroxy or carboxyl functions although a small amount of these cannot be excluded by the spectrum. These indications of several functional chemical groups, if all derived from a single molecule, suggest a fairly complex molecule.

Elementary analyses. — The ultramicroanalysis (6.7) made with a single ca. 0.5 mg. sample gave: C, 61.6 (± 1.5) %; H, 8.8 (± 0.3) %; N, 3.4 (± 0.3) %; and O, by difference, 26.2 %. The empirical formula is, then, $C_{21.1} H_{35.9} O_{6.7} N_1$ or, in even integers $C_{21} H_{36} O_7 N$ with a minimum molecular weight of 414.

If this is, indeed, the composition of the molecule it fulfills the requirement for oxygen imposed by the infra-red analysis and is of sufficient size to include the various functions inferred to be present.

Mass spectrum. — The mass spectrum displayed no very distinctive characteristics. It is of interest, however, that the peaks at $m/e=31$, 44, and 58, which can, but do not necessarily, indicate fragments derived from methoxy, aldehyde, and unsubstituted ketone ($-\text{CH}_2\text{COCH}_2-$) groups, are significantly stronger than from molecules not containing such functional groups. These are the same types of functions indicated by the infra-red spectrum. The peak with the highest m/e occurred at 386. If this represents the ionized unfragmented molecule it is of the same order of magnitude as the minimum molecular weight indicated by the elementary analysis. Finally, the spectrum gave no evidence of aromatic rings.

The preceding analyses, taken one by one, are of doubtful validity. Taken together they reinforce rather than cancel out inferences. It seems likely on the basis of the fractionation procedure and the biological activity of the sample analyzed that the dominant molecular species was sirenin and the impurities present small in amount. The molecule, in summary, would appear to have a molecular weight of the order of 400, to be nonaromatic and free, or almost free, of hydroxy and free carboxyl groups. It would appear to contain a lactone ring, ketone and aldehyde groups, and perhaps methoxy groups.

Discussion

The purification procedure described, or others that can be derived from it, makes possible the preparation of pure samples of sirenin. The analytical information suggests that the molecule is chemically complex thereby probably making the determination of the molecular structure a difficult venture and the production and purification of adequate amounts of sirenin a sizable undertaking. Although sirenin, so far as is known, affects only one step in the sexual reproduction of but a single subgenus of organisms, the determination of its structure would begin to fill the gap that now exists in plant physiology on the biochemistry of the sexual reproduction of plants.

Summary

The female gametes of the water molds in the subgenus *Euallomyces* release into the surrounding aqueous medium a substance — earlier named *sirenin* — which attracts the male gametes. Aqueous solutions of sirenin, con-

taining on the average 50 to 100 $\mu\text{g.}$ per liter, were prepared by permitting gamete formation and discharge from female plants to take place into water. After freeing the solutions of gametes by filtration through Seitz filters, the sirenin was adsorbed on charcoal, eluted with chloroform, reextracted from the chloroform-free residue with water, and, after removal of the water, taken up again in chloroform. This partially purified material was fractionated on a silicic acid column. The final preparation caused a minimally detectable response in the bioassay at a concentration of 10 $\mu\text{g.}$ per liter.

Elementary, ultra-violet, infra-red, and mass spectrometric analyses made on a single sample of unknown but presumably high purity suggest the molecule to have a molecular weight of about 400 with an approximate formula of $\text{C}_{21}\text{H}_{36}\text{O}_7\text{N}$. It appears to be neutral and nonaromatic and to contain a lactone ring as well as ketone, aldehyde, and methoxy functions.

This research was done during my tenure as a Guggenheim Fellow while on sabbatical leave from the Department of Botany, University of California, Berkeley, California and was supported, in part, by a research grant (G 1291) from the National Science Foundation.

I am indebted to numerous individuals at the University of Uppsala, Uppsala and the Karolinska Institutet, Stockholm for generous advice and assistance. Specifically, I wish to acknowledge: Professor Nils Fries, whose guest I was in the Institute of Physiological Botany; Professor Arne Tiselius, who made available the facilities of the Institute of Biochemistry; Dr. A. Rosenberg who made and interpreted the infra-red spectra; Professor P. E. Lindahl and his staff, who made the ultra-violet spectra; Professor E. Stenhagen and Mr. R. Ryhage, who made and interpreted the mass spectra; Mr. W. Kirsten of the Microanalytical laboratory of the Institute of Medical Chemistry, who made the elementary analysis; and to Bengt E. Jonsell who served capably as my research assistant.

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Studies on the Metabolism of *Merulius lacrymans* (Jacq.) Fr.

I. Carbohydrate Consumption, Respiration and Acid Production in Surface Cultures

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Our knowledge of the metabolism of *Merulius lacrymans* (and further, the group of wood-decaying Hymenomycetes to which it belongs), is rather scanty. Only a few respiratory studies have been made (Zoberst 1952). The studies of acid production carried out by the F. F. Nord group on *M. lacrymans* and related species has led to conclusions (Smith 1949) which seemed to justify a reinvestigation of this problem, supported by respiratory measurements.

In the present paper experiments are reported in which the relationship between respiration and acid production has been studied, and in which also the consumption of carbohydrates has been followed, and related to the mycelial growth and metabolic events.

Materials and Methods

The fungal strains. — Two *M. lacrymans* strains were used, both from Centraal-bureau voor Schimmelcultures, Baarn, Holland. The strains are named Falck and Cartwright. They are somewhat old in culture, but possessed a satisfactory growth rate with seemingly normal mycelium. Both attacked cellulose and wood; the wood with a normal decay picture. The cultures were kept on malt agar prior to the growth studies reported here.

Media and inoculation. — In a preliminary experiment the cultures were grown in a medium containing carbohydrate, urea, potassium phosphate, magnesium sulfate,

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and thiamine. The growth was then not quite satisfactory. Besides, the substrate gave precipitation by autoclaving. In the final experiment, urea was substituted by peptone. The composition of the medium was as follows:

Carbohydrate	:	15.0 g.
Peptone (Difco)	:	3.0 g.
KH_2PO_4	:	1.5 g.
$\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$:	1.5 g.
Thiamine	:	1 mg.
Distilled water	to	1000 ml.

The carbohydrates used, were in the following five series: A, xylose only; B, equal weights of xylose and glucose; C, glucose only; D, equal weights of glucose and cellobiose; E, cellobiose only.

10 ml. of the substrate was transferred to each 100 ml. Erlenmeyer flask, previously weighed, so that evaporation during the autoclaving and growth period could be determined and compensated for. The flasks were covered with aluminium caps made from "Höyang" housekeeping aluminium foil. After autoclaving, the flasks were inoculated by small pieces of mycelium grown on malt agar. The cultures were kept in a constant temperature room of 22—23°C during growth.

Respiration measurements. — It was considered of importance to measure the respiration without disturbing the mycelium. With *M. lacrymans* surface cultures, this is a crucial point, since the aerial mycelium so easily collapses. Besides, on cutting the mycelium, reddish-brown spots are seen to occur, indicating the liberation of an oxidase system.

Therefore, the Warburg technique had to be abandoned. Instead, respiration measurements were carried out by gas analysis. A suitable period of time before the cultures should be taken for analysis, the culture flasks were weighed and water added to make up for that evaporated. The flasks were then flushed with fresh air for one minute, and stoppered by a rubber stopper through which passed a single-bore stopcock. The upper limb was cut about 1 cm. above the cock and ground to fit the rubber cap of the gas transfer syringe. Gas analysis was then carried out by means of a Scholander $\frac{1}{2}$ ml. gas analysis apparatus. After a period during which the flasks were kept with closed stopcocks in the constant temperature room (22—23°C), varying between 72 and 24 hours, the air in the flasks was analyzed anew. The total amounts of oxygen consumed and carbon dioxide evolved were calculated on basis of the total gas volume in each flask, and the amount of gas dissolved in the substrate. The total gas changes were finally reduced to normal conditions.

Carbohydrate analyses. — In all series, total carbohydrate content was determined according to the method of Nelson (Bell 1955) on 0.04 ml. samples taken out by a Carlsberg pipette. Triplicate analyses were carried out in each flask. The amounts of the various carbohydrates were determined in the flasks from the series B, D, and E by separation on filter paper (Whatman filter paper no. 1, system: Ethyl acetate: acetic acid: water 3 : 1 : 3) and eluting the paper where the sugar spots had been localized on parallel strips developed with aniline phthalate. In the eluate, sugar was determined according to the previously mentioned method.

Acid production. — pH measurements were made in the substrate at the end of the growth period. On five ml. samples titration with 0.05-N NaOH was carried out with glass electrode and pH meter to pH 8.5, and the difference between the titration value on the substrate at start and at the end of the growth period was taken as total acidity produced, calculated in milliequivalents.

Table 1. *The strains have been taken for analysis as indicated by crosses.*

Weeks after inoculation	1	2	3	4	5	6	7	8	9	10
Falck.....	×	—	×	—	×	—	—	×	—	×
Cartwright	—	×	—	×	—	—	×	—	×	—

Acid-base indicator could not be used in this titration, owing to the yellow colouring matter formed by *M. lacrymans* in the substrate, acting in itself as an acid-base indicator (Zoberst 1952). After titration, the solution was brought back to the Erlenmeyer flask, the mycelium filtered away, washed, dried and weighed, and oxalic acid precipitated in the filtrate as calcium oxalate by addition of calcium chloride to the solution made acid with HCl to a pH of about 4.5. The precipitate was centrifuged, washed and redissolved in sulfuric acid and finally titrated with 0.05-n potassium permanganate.

Results

Owing to the number of analyses carried out on each flask, only one flask from each series could be analyzed a week. The analyses alternated between the Falck strain and the Cartwright strain as shown in Table 1.

The strains behaved very similarly, and it does not seem possible to differentiate between them on the basis of the results presented here.

In Figure 1 are shown the dry weights of the mycelia in the different series, at the times when analyses were made. The fact that there is only one

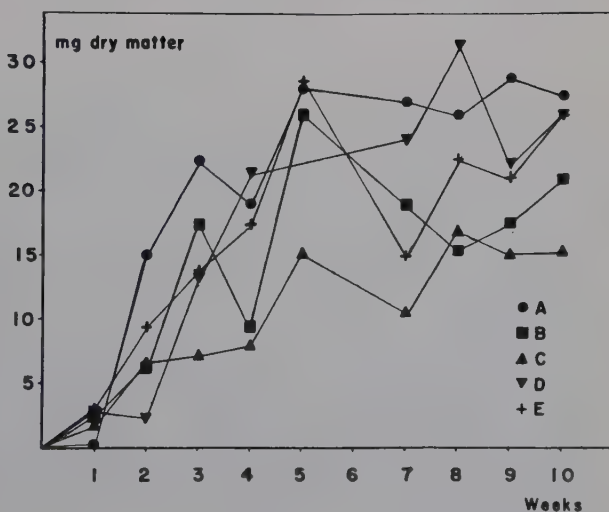


Figure 1. *Dry weights of the mycelia in the five series (ordinate) in relation to the growth period (abscissa). The individual values in each series have been interconnected by straight lines in order to obtain clearer picture.*

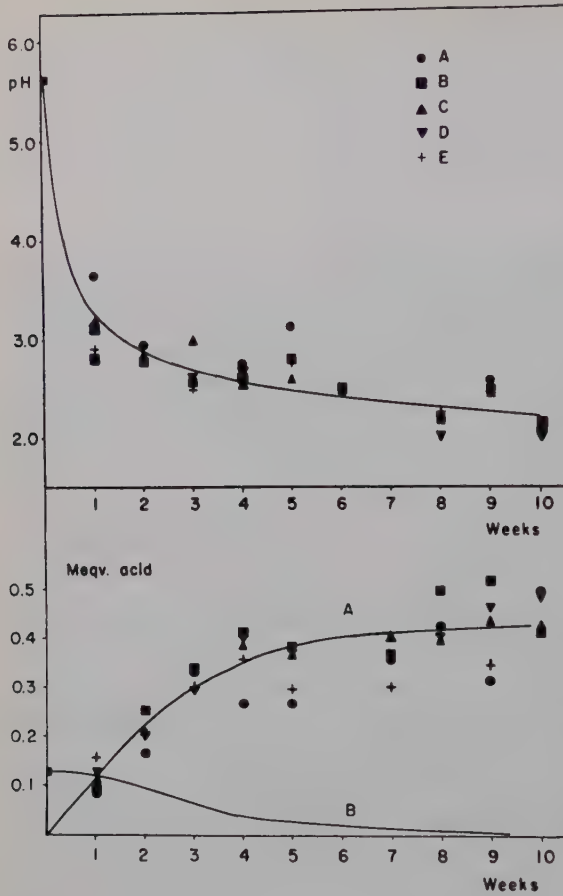


Figure 2. Upper diagram; the changes in pH during the growth period. Lower diagram; symbols and curve A the total amount of acid produced, curve B, the mean amount of acid produced per week, both in milli-equivalents. Abscissa; the growth period in weeks.

dry weight determination behind each value accounts for the great variations. The values indicate that better growth is obtained with xylose than with glucose. This is in agreement with Zoberst's findings. The growth seems to have practically stopped after 5 weeks. Beyond this the results do not permit any certain conclusions.

In Figure 2 are shown the changes in pH values during growth. It is seen that pH falls rapidly at start, and is approaching 2.0 at the end of the experimental period.

In Figure 3 is shown the decrease in carbohydrates in the five series, related to the mycelium dry weights.

Series B shows that in a mixture of xylose and glucose, glucose is preferentially utilized, xylose only being used to a slight extent when glucose is present. When the series A and C are compared, it is seen that more glucose than

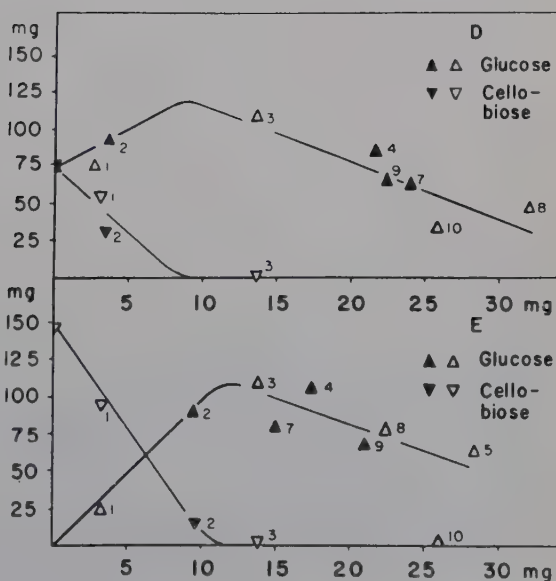
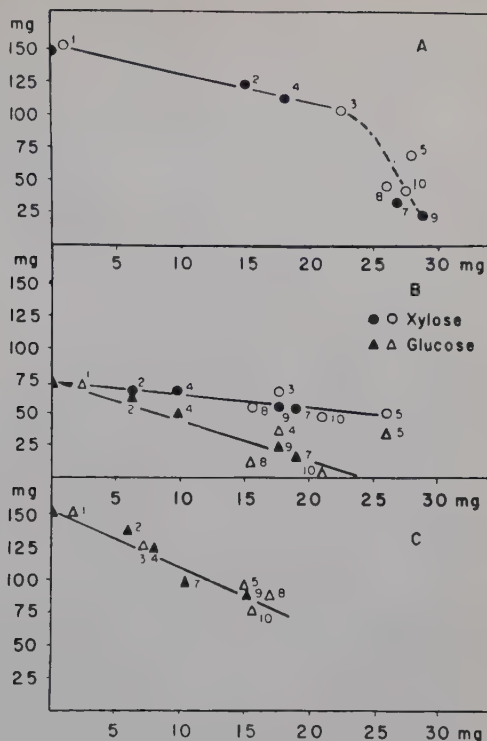


Figure 3. The amounts of carbohydrate found in the culture substrates (ordinate) in relation to the mycelial dry weight (abscissa). Open symbols; the Cartwright strain, closed symbols; the Falck strain. The figures given beside each symbol are the periods of growth in weeks. Figure A shows the results from series A, figure B from series B, etc.

Table 2. *The economic coefficient* (mg. mycelium produced per mg. carbohydrate consumed). Results from the series A (first period of growth), series C, and from the glucose consumption in series D and E.

Series	Carbohydrate	Ec. coefficient
A	Xylose	0.55
C	Glucose	0.26
D	"	0.28
E	"	0.32

xylose is used for obtaining a certain mycelium weight. In series A, however, the curve has a break after about 24 mg. dry weight. This corresponds roughly to the cessation of growth in this series.

Series D and E show that cellobiose is rapidly hydrolyzed, making it difficult to conclude whether this carbohydrate is utilized as such or not. The utilization of glucose gives about the same economic coefficient (mg. mycelium dry weight/mg. carbohydrate consumed) as in series C (Table 2). In Figure 4 is shown the respiratory activity measured as Q_{O_2} (microliters oxygen consumed per mg. and hour) in relation to mycelial dry weight. The figure shows that all series have the same respiratory pattern: A relatively high Q_{O_2} value in the young cultures, probably lying around 10 by the time of inoculation, and gradually decreasing to about 1.

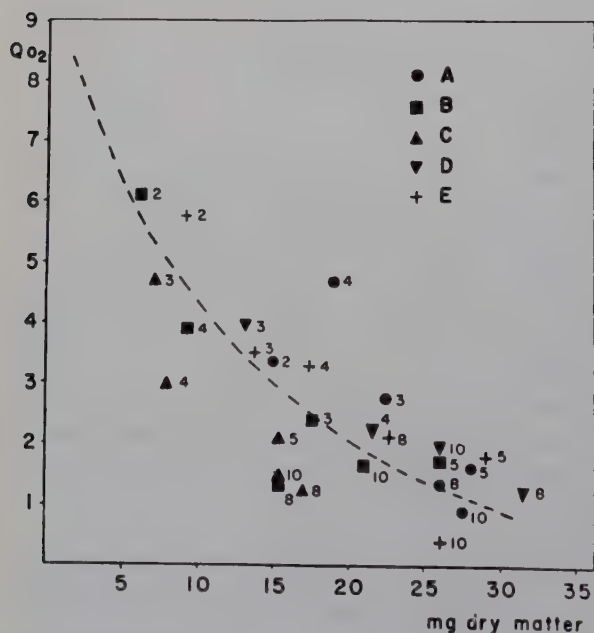
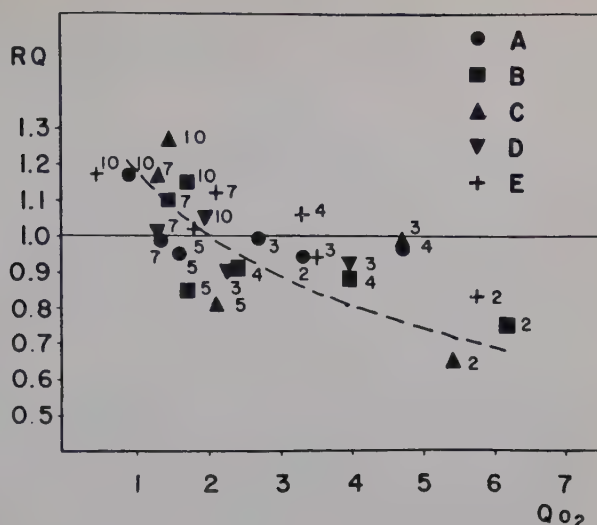


Figure 4. *The oxygen consumption per mg and hour (ordinate) in relation to the mycelial dry weight (abscissa). The figures beside each symbol are the periods of growth in weeks.*

Figure 5. *The respiratory quotient (ordinate) in relation to respiratory intensity (abscissa). The figures beside each symbol are the periods of growth in weeks.*



The respiratory quotient, RQ, also changes, as is shown in Fig. 5. Here RQ is related to Q_{O_2} , and from the single values it can be seen that the young cultures with a high Q_{O_2} have a low RQ, whereas the older ones with a low Q_{O_2} have a RQ around or above 1.

The acid production shows a picture resembling that of the respiratory intensity. This is shown in Figure 6 where acid production per mg. dry myce-

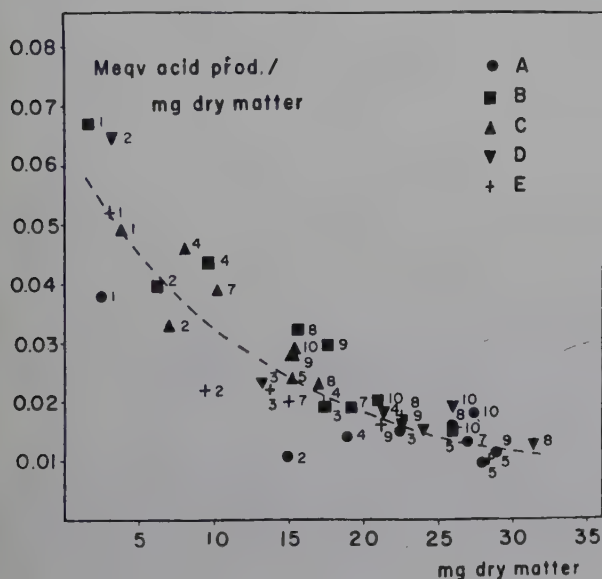


Figure 6. *The total acid production in milliequivalents (ordinate) in relation to the mycelial dry weight (abscissa). The figures besides each symbol are the periods of growth in weeks.*

Table 3. *The amounts of oxalic acid produced in per cents of total acid during the growth period.*

Weeks after inoculation	Series				
	A	B	C	D	E
3	91	95	—	96	99
5	—	90	93	—	97
7	99	95	101	94	113
8	93	99	100	89	96
9	112	80	105	104	107
10	92	96	100	84	101

lium is related to the mycelium dry weight. A nice correlation is found, showing how the acid production capacity of the mycelium decreases with time and with increasing amount of mycelium.

Table 3 shows the amounts of oxalic acid as per cent values of total acidity during the culture period. The mean value, 98.2 % shows that very little of other acids are formed, as compared to oxalic acid.

Discussion

It may be pertinent to quote a passage from Smith (1949): "With this mold (*Merulius lacrymans*), oxalate is the end-product corresponding to CO₂ in some organisms, and is apparently utilized only when glucose levels are low".

The oxidation of a hexose to oxalic acid and water would give the following over-all formula:



The RQ of a respiration based on this reaction would of course be zero. If no fermentative processes did occur, it would be possible to calculate the ratio between normally respired hexose (to carbon dioxide and water) and that oxidized to oxalate, at a certain RQ.

It can for instance be found that at an RQ of 0.7, the lowest observed in these studies, 64 % of the glucose should be respired normally to carbon dioxide and water, and 36 % oxidized to oxalic acid.

Fermentation processes causing liberation of CO₂ would of course, if occurring simultaneously, invalidate such a calculation. It might well — on the paper, at least — be possible to obtain any RQ if the two processes, oxidation to oxalate and *e.g.* fermentation to ethanol, occurred side by side in varying ratios.

Since fermentation products (ethanol) have not been determined in our cultures, there is no direct opportunity of proving or disproving such a possibility. It can, however, be checked indirectly, since oxalic acid production has been determined.

We may first consider the respiration and acid production at the start of the growth period. Assuming a Q_{O_2} of 10 and RQ of 0.7, we will then have a consumption rate of 75 μ -moles O_2 and an evolution rate of 52.5 μ -moles CO_2 per week per mg. dry mycelium. Or, if linear growth rate is assumed, and it is calculated with a mean weight of 2.5 mg. after a week, 94 μ -moles O_2 have been consumed and 65 μ -moles CO_2 evolved during the first week. The mean amount of acid produced during the first week is 120 μ -equivalents. This corresponds, if all acid is calculated as oxalic acid, to 90 μ -moles of oxygen. Thus, virtually no oxygen is free for normal respiration of hexose, and accordingly practically all the 65 μ -moles of CO_2 must origin from a parallel fermentation process. Thus, it seems that in fact oxalic acid is the principal end product of the aerobic part of respiration during the first phase of growth in our cultures.

It is seen from Figure 2 that acid formation declines after the third week of growth. At the same time, RQ approaches 1. The amount of acid formed per week from the fifth week onwards, is roughly 0.01 m-equivalents, or even more roughly, 0.5 μ -equivalents oxalic acid per week per mg. mycelium. This corresponds to about 0.4 μ -moles oxygen per week and mg. A Q_{O_2} value of 1 would mean that 7.5 μ -moles of oxygen is consumed per week and mg. Thus, at this stage of growth, normal respiration of carbohydrate to CO_2 and water must account for the major part of the oxygen uptake. But the RQ values above 1 indicate that fermentation processes still occur.

It seems difficult, from the results obtained here, to get a deeper insight into details of the respiratory and fermentative processes in *M. lacrymans*.

Studies are now under way, with the intention of elaborating some of these details.

Another point of interest is found in the utilization of different carbohydrates for growth. In agreement with the findings of Zoberst (1952), growth is considerably better on xylose than on glucose. The peculiar thing is, however, that whereas xylose is more economically utilized than glucose, it is the latter carbohydrate which is preferentially removed from the substrate.

An explanation near at hand which might find support in the results, although the data are somewhat scarce and not quite clear, is that with glucose, the acid production is higher than with xylose, resulting in a more rapid decrease in pH and consequently more rapid development of unfavourable conditions for growth. It is seen from Figure 2 that the pH values for the xylose series have a slight tendency of lying higher than for the glucose

series. This becomes more pronounced when the mycelium dry weights are brought into the picture, and Figure 2 is compared with Figure 1. The ratio acid production/mycelium production is in the glucose series more than twice what it is in the xylose series during the first 3—4 weeks of the growth period.

Summary

Growth experiments have been carried out with two strains of *Merulius lacrymans*, viz. the Falck strain and the Cartwright strain from CBS, Baarn, Holland. The fungi were grown as surface cultures on a liquid medium consisting of carbohydrate, peptone, potassium phosphate, magnesium sulfate and thiamine. As carbohydrates were used xylose, a mixture of xylose and glucose, glucose, a mixture of glucose and cellobiose, and finally, cellobiose.

Growth, total acid production, oxalic acid production, carbohydrate disappearance, respiratory intensity and respiratory quotients were determined every week during the growth period, which lasted 10 weeks.

The two strains behaved very similar, and from the data it does not seem possible to distinguish between them.

Growth was better on xylose than on glucose, while the other series were intermediary. Cellobiose was rapidly split to glucose. In a mixture of xylose and glucose, the latter was utilized while the xylose was retained.

The economic coefficient with glucose (and also cellobiose and cellobiose-glucose) was about 0.25—0.30 mg. mycelium per mg. carbohydrate consumed, while it was about 0.55 with xylose.

The respiratory intensity was high at start of the growth period, probably with Q_{O_2} -values around 10 at the time of inoculation. At the same time, RQ was low, around 0.7. Q_{O_2} decreased gradually to values of 1—2, while RQ increased to values well above 1.

The acid production during the first week of growth was so high as to account for all the oxygen consumed, indicating that the dominating respiratory process during this period resulted practically only in the formation of oxalic acid, and consequently, that the carbon dioxide must have been formed mainly from a fermentative process, going on simultaneously.

At the later stages of growth, the acid production could only account for a small fraction of the amount of oxygen consumed, and a gradual shift to normal aerobic respiration is thus indicated. The concurrent increase of RQ beyond the corresponding limit (ca. 0.9) shows that fermentative processes must occur at this stage also.

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Induction Phenomenon and CO₂ Gush in Photosynthesis of *Polytrichum attenuatum*

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It was shown previously (Vejlby 1958) in a study of the induction phenomenon in the moss *Polytrichum attenuatum* that curves depicting the connection between the time and the amount of carbon dioxide taken up during the process of photosynthesis (the time curve) are essentially in agreement with corresponding curves for higher plants; the most conspicuous difference observed was that the moss curves exhibit only one induction peak 30 to 60 seconds after the onset of photosynthesis, whereas two peaks or more often are found in experiments with other plant groups (Aufdemgarten 1939, van der Veen 1949). Also, it was shown that the size of the induction peak in mosses increases with increasing concentrations of carbon dioxide and light intensities, but decreases with increasing temperatures; it disappears at 30°C, only to reappear in subsequent experiments at lower temperatures.

A series of experiments with intermittent illumination interrupted by shorter or longer dark periods were carried out in an attempt to analyze the induction phenomenon more thoroughly. The results of this study justify a new interpretation of the course of reactions during the initial phases of photosynthesis.

Material and Methods

A number of the specimens of *Polytrichum attenuatum* Menz. used were collected in Rude Forest, as was the case in the previous study, and the rest were taken in Tisvilde plantation in northernmost Zealand. No differences in the reactions of these

plant materials were observed. The plants were kept in covered glass dishes placed in an air thermostat at 10°C. The plants were regularly supplied with water to keep their turgor at a constant high level. The illumination was kept constant at an intensity of about 50 cal (λ 400—700 m μ)/dm². hr. (~1750 lux).

The experimental arrangement was exactly as before, the diaferometer method being used for present experiments as well. Five to ten moss plants corresponding to a fresh weight of 0.25 g. were used for each individual experiment. The temperature of the experiment was 14°C, the carbon dioxide concentration was 3.1 vol. per cent, and the velocity of the air flow was approximately 3 litres per hour. Before the start of the experiments proper the sample plants were invariably placed in the dark in 3.1 per cent CO₂ for about 1 hour. A light intensity of 75 cal. (λ 400—700 m μ)/dm.² hr. (~2600 lux) was used for the experiments. The light source was a 2000 watt incandescent lamp placed in a running water bath.

The light intensities were determined by means of a selenium barrier photocell with neutral filters. The photocell was calibrated in absolute units valid for the spectral range of 400 to 700 m μ . The conversion into lux was made according to a determination by Gabrielsen (1948) showing 28.6 cal (λ 400—700 m μ)/dm.² hr. to correspond to 1000 lux for a 1500 watt Osram=nitra lamp.

Results

Van der Veen (1949) previously published a study of the induction phenomenon in intermittent light, but included only one combination type of light and dark periods, *i.e.*, 3 minutes of illumination followed by a 2 minute dark period, this sequence being repeated a total of three times. In these experiments the peak of the time curve was already fully developed before the onset of the first dark period. It would therefore appear to be of interest to study the phenomenon during considerably shorter intervals of light and dark.

In Figure 1 are shown the results of two experiments, the light period being in the one case an uninterrupted 17 minutes (curve A), while in the other case 15 seconds of preliminary light was followed by a 1 minute dark period, this combination being repeated 3 times and being subsequently followed also by an uninterrupted light period of 17 minutes (curve B). In the figure the periods of light and dark are indicated by areas of light and dark under the curves. Curve A has the usual appearance while in curve B the primary peak has been transformed into four considerably smaller ones followed by a greater, fifth peak after the onset of the longer period of light. It is worth noting that during the dark period following each 15 seconds light period curve B drops below the axis of abscissa which can be interpreted only as a registration of an output of carbon dioxide. Immediately on the onset of another light period the curve rises instantly and this rise continues for an additional 15 seconds into the subsequent dark period.

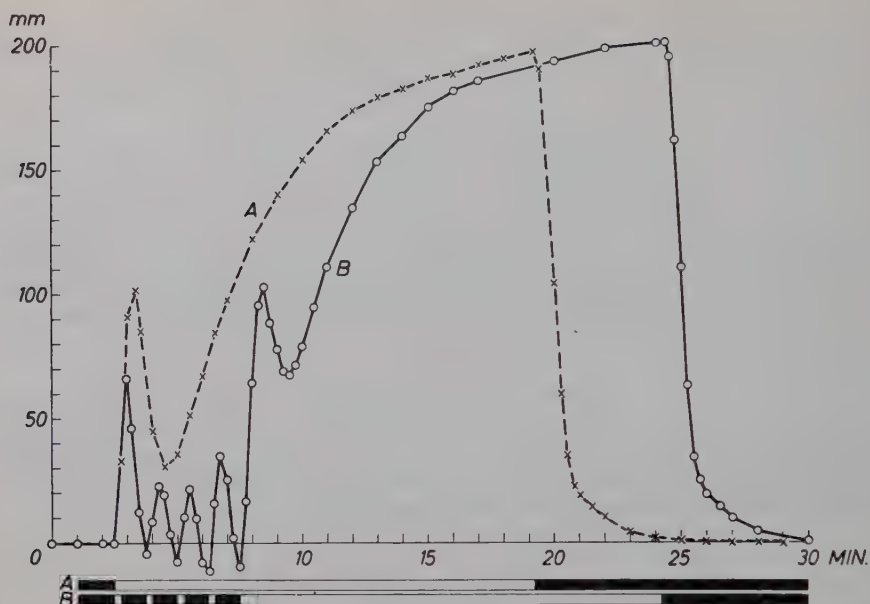


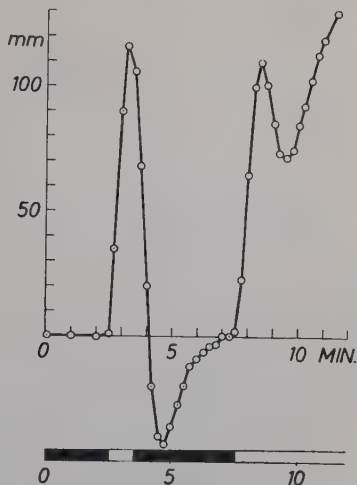
Figure 1. Photosynthetic time curve for 0.25 g. mossplants in 3.1 % CO_2 . Light intensity $75 \text{ cal/dm}^2 \text{ h}$. Temperature 14°C . Curve A: 17 minutes light. Curve B: $4 \times \frac{1}{4}$ minutes + 17 minutes light. Abscissa: time in minutes. Ordinate: Galvanometer deflection in mm.

If the experiment is altered to include a total of 10 flashes of light, each lasting 15 seconds and each followed by a one minute dark period, the course of the time curve is in principle identical to that of Figure 1 B. The primary peak is somewhat smaller than that obtained by uninterrupted illumination, and the nine subsequent peaks in turn are each smaller than the primary one, but the nine of them are approximately identical in size. Finally, upon the onset of the long light period, yet another peak is registered, this one being of the same order of magnitude as the one appearing upon a direct change to uninterrupted illumination.

If the experiment corresponding to curve B (Figure 1) is carried out in such a way that a quantity of light equal to that of the four periods is given as one dose in a light period lasting one minute, followed by a 4 minute dark period, the time curve assumes the course shown in Figure 2. It is obvious from this figure (the great CO_2 gush) that the uptake of carbon dioxide, which commences simultaneously with the onset of illumination, after 45 seconds of illumination is apparently replaced by, or possibly runs parallel to, an evolution of carbon dioxide.

To further elucidate the reason for the occurrence of the CO_2 gush a series

Figure 2. *Detail of photosynthetic time curve for 0.25 g. mossplants in 3.1 % CO₂. 1 minute light and 4 minutes dark followed by light. Abscissa: time in minutes. Ordinate: Galvanometer deflection in mm.*



of experiments was carried out, four of which are reproduced here in Figures 3 A, B, C, and D. The preliminary light period lasted 30 seconds, and 2, 3, and 4 minutes, respectively, and the subsequent dark period was in all cases 4 minutes.

In the experiment 3 A (Figure 3 A) the initial light period is interrupted at a time corresponding to a nearly fully developed induction peak just before the time curve starts its decline. In Figure 3 B the interruption is undertaken at a time when the curve, in experiments with continuous light, normally shows a minimum. In Figure 3 C the dark interval sets in one minute later, at a time when the curve has already started its second upward trend; finally, in Figure 3 D, we may observe the effect of the insertion of a dark period at the moment when the second upward trend has reached half of the peak value. In all of these experiments a CO₂ gush is observed, and it is evident that the apparent suspension of the uptake of carbon dioxide occurring after 45 seconds of illumination is due to a reverse process involving an evolution of carbon dioxide, which becomes observable at this very time and which under the conditions of the experiment lasts approximately 3 to 4 minutes.

If the initial light period is extended to last a total of six minutes, the evolution of carbon dioxide appears to have ceased (Figure 4 B). But in this curve as well as in the curves in Figures 3 A, B, C, and D an additional characteristic fact is the occurrence of yet another induction peak during the continuous light period following the dark interval.

In order to study the reasons for this secondary induction peak, experiments were made using even longer initial light periods than before. The

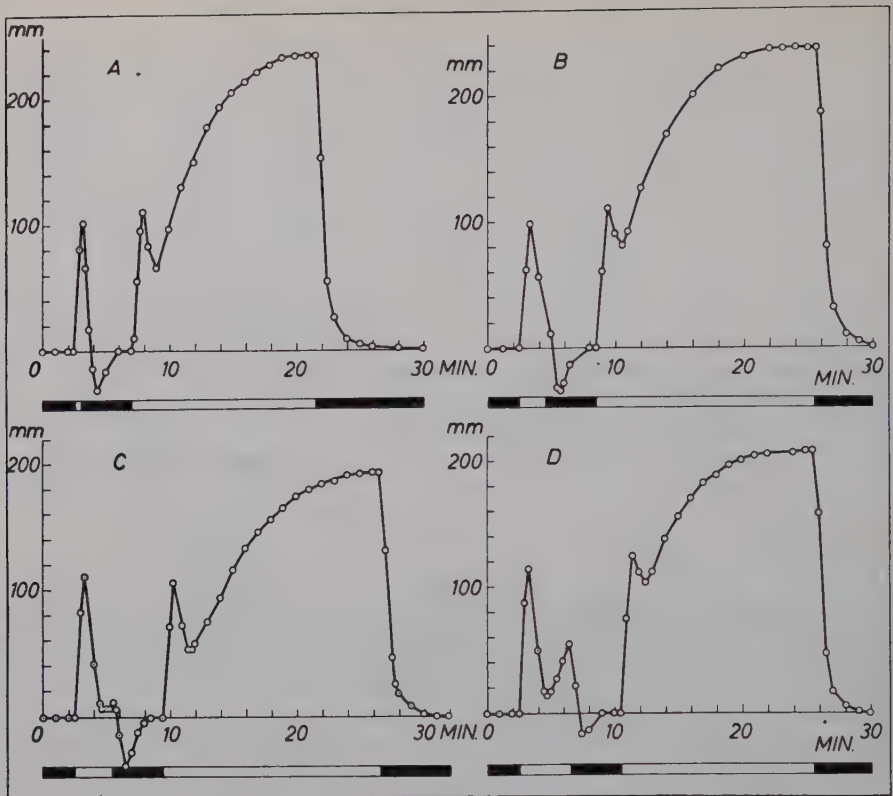


Figure 3. Photosynthetic time curves at different lengths of the primary light period. A: $\frac{1}{4}$ minute. B: 2 minutes. C: 3 minutes. D: 4 minutes. The following dark period is in all four cases 4 minutes. Abscissa: time in minutes. Ordinates: Galvanometer deflections in mm.

peak still occurred even when a 12 minute light period was adopted. When, on the other hand, the duration of the inserted dark period is reduced from 4 minutes to 30 seconds, no secondary peak occurs during the subsequent light period (Figure 4 A).

This might indicate the existence during a 4 minute dark period of a process causing an evolution of carbon dioxide during a subsequent light period. However, if the dark period is reduced to 30 seconds, this appears not to be the case. If the duration of the dark period is 45 seconds this process determining the subsequent evolution of carbon dioxide appears to take place indeed, although not to any great extent (Figure 5, cf. Figure 2).

The experiments described in this paper show photosynthesis in *Polytrichum attenuatum* to start with a vigorous uptake of carbon dioxide lasting

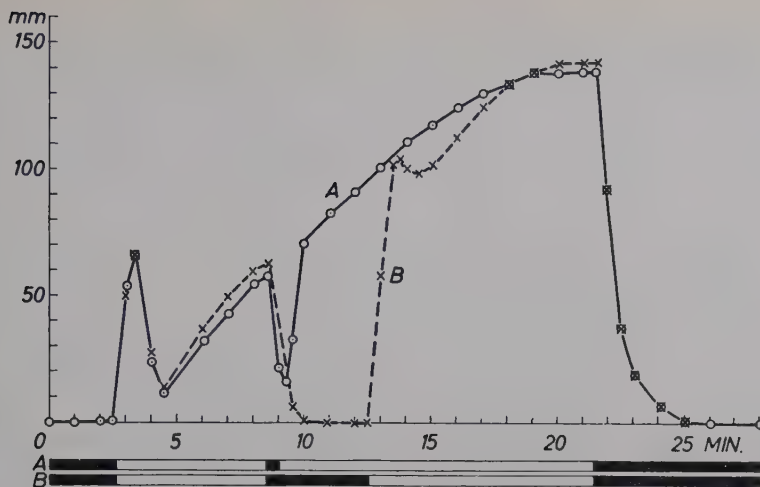


Figure 4. *Photosynthetic time curves at different dark periods following a primary light period of 6 minutes. Curve A: $\frac{1}{2}$ minute dark. Curve B: 4 minutes dark. Abscissa: Time in minutes. Ordinate: Galvanometer deflection in mm.*

approximately 45 seconds and being then replaced by an evolution of carbon dioxide of a duration of approximately three and a half minutes.

After a secondary dark period of 4 minutes and subsequent illumination the time curve shows an additional peak, probably caused by a second evolution of carbon dioxide possibly of somewhat shorter duration. If the sec-

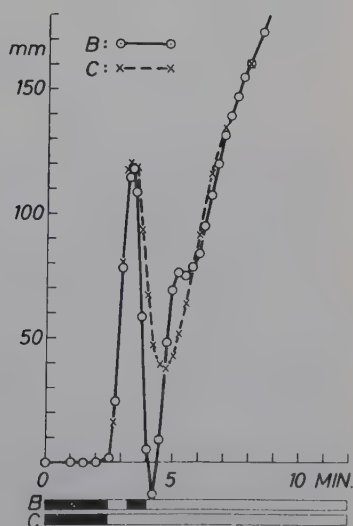


Figure 5: *Detail of photosynthetic time curves. Curve B: $\frac{3}{4}$ minute light and $\frac{3}{4}$ minute dark. Curve C: Continuous light. Abscissa: time in minutes. Ordinate: Galvanometer deflection in mm.*

Table 1. *Survey of the results.*

Number and duration of		Observation of		Figure
preliminary light periods	dark periods	indisputable CO ₂ gush	number of peaks ¹	
0	0	no	0 + 1	1 A
4 × 1/4 minute	4 × 1 minute	yes	4 + 1	1 B
1 × 1 minute	1 × 4 minutes	yes	1 + 1	2
1 × 1/4 minute	1 × 4 minutes	yes	1 + 1	3 A
1 × 2 minutes			1 + 1	3 B
1 × 3 "			2 + 1	3 C
1 × 4 "			2 + 1	3 D
1 × 6 minutes	1 × 1/2 minute	no	1 + 0	4 A
1 × 6 "	1 × 4 minutes		1 + 1	4 B
1 × 3/4 minute	1 × 3/4 minute	yes	1 + (1)	5 B

¹ Number of peaks caused by the preliminary light periods + number of peaks occurring in the long final light period.

ondary dark period is reduced to 30 seconds the time curve shows no secondary peak during the subsequent light period; but a secondary dark period lasting 45 seconds causes a small shoulder to appear on the time curve approximately one minute after the onset of the long period of illumination. A comparison of the results is found in Table 1.

In experiments with short light periods (15 seconds) the uptake of carbon dioxide appears to continue for 15 seconds after the onset of the subsequent dark period.

Discussion

Induction phenomena including an evolution of carbon dioxide have previously been described on the basis of experiments with unicellular algae (*Chlorella*) by Emerson and Lewis (1939, 1941) and confirmed by Emerson and Nishimura (1949) and by Emerson and Chalmers (1957). Subsequently van der Veen (1950) using the diaferometer method was likewise able to record a CO₂ gush in *Chlorella*, and Brown and Wittingham (1955) further confirmed this by means of mass spectrometer experiments and showed the existence of a similar phenomenon in *Scenedesmus* cells.

Recently Massini (1957) studied induction phenomena in higher plants (*Datura*, *Dahlia*, *Impatiens*) by determining the uptake of radioactive carbon dioxide during illumination periods of short duration. He finds that the car-

bon dioxide taken up during the first minute of illumination is not fixed in a stable, extractable compound, and explains this fact by assuming the accelerating formation of energy rich phosphates produced by the illumination (Arnon 1956) to cause an increase in pH, which then in turn causes an uptake of CO₂. If this explanation is accepted the CO₂ gush in the experiment corresponding to Figure 2 in this paper may possibly be explained as resulting from the reverse of the above process, which may be expected to set in upon the cessation of illumination, and before the initial uptake of carbon dioxide has ceased. On the other hand, this assumption fails to explain the course of curve A in Figure 4, where no peak is found following the secondary 30 second dark period. Nor do the previously published temperature experiments (Vejlbj 1958), in which the primary peak was found to disappear at 30°C, but where the initial increase of the time curve is identical at 13° and at 30° appear to confirm the assumptions of Massini. Finally, it may be noted that Blinks and Skow (1938) in a study of sea algae as well as leaves of fresh water plants and land plants found even very short flashes of light to produce a change of the pH in the leaves, the change being, however, towards the acid part of the scale.

Until now ribulose diphosphate has been considered the primary acceptor of carbon dioxide in photosynthesis, but recently Metzner, Simon, Metzner and Calvin (1957) published a series of experiments with the green alga *Scenedesmus*. These experiments show that in this plant, at least, another acceptor of carbon dioxide is found in addition to ribulose diphosphate, and that the product formed from this acceptor and CO₂ is unstable. The presence of this compound has been demonstrated in the cells of the algae following photosynthesis or dark fixation of radioactive carbon dioxide. The unstable CO₂ compound reaches its maximum concentration after approximately 2 minutes of photosynthesis, but is partly decomposed under the formation of carbon dioxide, while the residue is converted into stable products, mainly phosphoglyceric acid. Further, the experiments show the unstable CO₂ compound to be even more labile at higher temperatures, and this is one of the reasons why previously, when the algae were killed in hot ethanol after photosynthesis experiments, the presence of this compound could not be demonstrated.

It seems as if these experiments, in combination with the present results, may provide an explanation of the induction phenomena. Figure 6 shows the "normal" time curve ABED. The primary peak ABE should then appear as a result of two reverse processes, one being the uptake of CO₂ caused by the onset of photosynthesis, and the other being the subsequent evolution of CO₂ caused by the decomposition of the primary, unstable CO₂ fixation product shortly after the onset of photosynthesis.

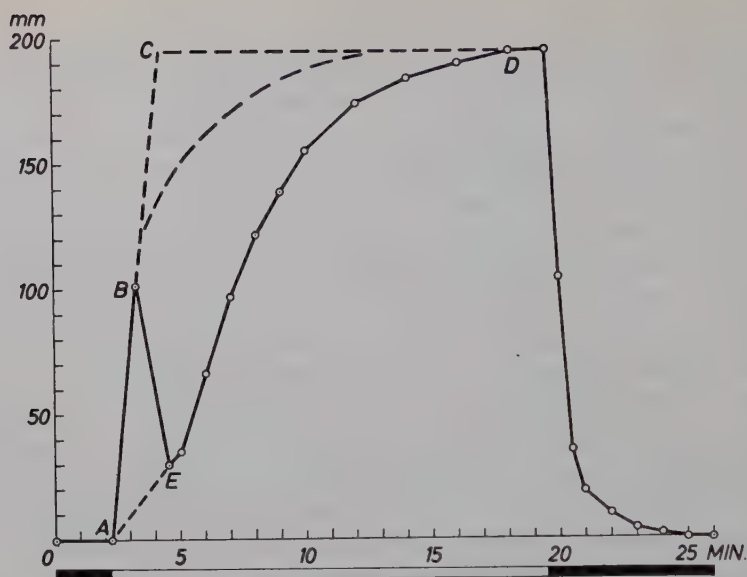


Figure 6. Photosynthetic time curve (continuous line ABED) together with some hypothetical sequences (dotted lines). Abscissa: time in minutes. Ordinate: Galvanometer deflection in mm.

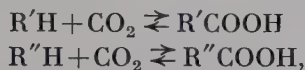
It might be assumed that had there been no formation of CO_2 the time curve would have followed the course ABCD; but since the formation of the carbon dioxide acceptor "proper", ribulose diphosphate, is a process which, as shown by Bassham, Shibata, Steenberg, Bourdon and Calvin (1956), proceeds (in *Scenedesmus*) over a period of 8 to 10 minutes, the course ABD appears to be the more likely one.

The observation of Emerson and Lewis (1939, 1941) of the evolution of carbon dioxide at the onset of photosynthesis was later interpreted by Franck (1942) who connected these observations with the theories advanced by Franck, French and Puck (1941) concerning induction phenomena and based partly on the opinions of Gaffron (1937). According to this view all of the abnormalities during the initial phases of photosynthesis are due to a temporary deficiency in an oxygen-removing catalyst, named catalyst C by Franck *et al.* A number of the comments made by Franck in 1942 are based on the observation by Emerson and Lewis that a change from a low to a high light intensity again gives rise to a formation of carbon dioxide, even after a long period of illumination at the low light intensity. In the experiments with *Polytrichum* this would correspond to the registration of an additional induction peak at the change from a low to a higher light intensity, but a phenomenon of this type has never been observed.

The present experiments would agree considerably better with the assumption that an unstable product of CO₂ fixation has been accumulated during the preceding dark period, and that after about 30 seconds of illumination this product starts decomposing with the formation of carbon dioxide. Further, the experiments indicate that during an inserted dark period lasting 4 minutes a certain amount of the unstable CO₂ product may again be formed, only to be decomposed anew during subsequent photosynthesis. If, however, the inserted dark period lasts only 30 seconds there appears to be no possibility of a formation of appreciable amounts of the above carbon dioxide compound (Figure 4).

It is difficult on the basis of the present material to decide whether this decomposition is a direct photochemical reaction which, perhaps due to diffusion, is not registered until after 30 seconds, or whether it is the case of a back reaction due to the onset of the photosynthetic assimilation "proper".

The following two reactions might be assumed to take place



R'COOH being the unstable product of CO₂ fixation found by Metzner, Simon, Metzner, and Calvin (1957), while R''COOH is the keto-acid assumed by Bassham, Benson, Kay, Harris, Wilson, and Calvin (1954) to be the precursor for the formation of phosphoglyceric acid. In this case R''H represents ribulose diphosphate, and as soon as this carbon dioxide acceptor starts functioning to any considerable extent, the concentration of CO₂ surrounding the chloroplasts will decrease, a decrease which in turn might cause a splitting of R'COOH to R'H plus CO₂. The previously mentioned temperature experiments, which in principle show the course of the curve (Figure 6) ABED at 13°C to change to ABD by heating to 30°, could then be interpreted to mean that R'COOH due to the change in temperature is not formed at all at 30°. This then would be in agreement with the experiments of Metzner et al. showing this compound to be exceedingly unstable at higher temperatures, since it is only possible to demonstrate the existence of this unstable product of CO₂ fixation when the sample plants are transferred to acetone or ethanol and cooled to -30°C after only a few seconds of photosynthesis.

Summary

The induction phenomenon during the initial uptake of carbon dioxide in the photosynthesis of the moss *Polytrichum attenuatum* Menz. was studied by means of the diaferometer method. When using intermittent illumination an evolution of carbon dioxide may be demonstrated 45 seconds to 1 minute

after the onset of illumination. However, this gush of CO_2 occurs only provided the plants have been placed in the dark for a minimum of 45 seconds prior to illumination.

It seems as if in the dark the plants accumulate carbon dioxide with the formation of an unstable product of CO_2 fixation, which decomposes forming carbon dioxide when the plants are exposed to light and photosynthesis sets in. It is possible that the induction phenomenon, to a certain extent at least, may be explained as being the result of this gush of CO_2 .

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Protochlorophyll in Root Tips

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This paper originates from the observation that root tips grown in darkness exhibit distinct red fluorescence when activated with strong blue light. In the literature nothing can be found on red fluorescence in roots in spite of the fact that fluorescing substances in this organ have been investigated many times (Linsbauer 1929, Goodwin and Kavanagh 1948, Goodwin and Pollock 1954 and Eberhardt 1955). These authors observed only white, yellow or blue fluorescence. However, they used UV light for activation. Only Goodwin and Kavanagh (1948) cursorily mentioned a red fluorescence in the root tip of young dark-grown seedlings of *Lupinus perennis* but concluded that it was probable not due to the presence of chlorophylls.

The subject of this paper is the nature and occurrence of the red fluorescing substance in the roots of plants.

This work has been performed by means of a simple fluorescence microscope made of a normal microscope by addition of a 1.5 cm. thick solution filter of ammonium copper sulphate as primary filter between lamp and microscope and a 1.5 mm. thick layer of saturated K_2CrO_7 or orange glass filter over the ocular as a secondary filter. A Zircon arc lamp served as a light source. It gave an intensity of light high enough to cause strong fluorescence of the chlorophylls as well as of the fluorescing pigment in root tips. Data on the material used and other methods will be mentioned below.

The Occurrence of a Red Fluorescing Substance

The first observation was made on wheat roots the growth of which was inhibited by 1-naphthyl-acetic acid. These roots exhibit strong red fluorescence in the innermost layer of the cortex of the mature parts when grown

in light. This is due to chlorophyll (Burström and Hejnowicz 1958). In roots grown in darkness, on the other hand, red fluorescence occurs in the very tips (cell division zone) of both primary and lateral roots. In the latter case red fluorescence appears as soon as the new meristem has been formed and before it emerges from the maternal root. The preliminary determination of the fluorescence light by means of interference filters gave the following results: the strongest activation is caused by light about 430 m μ and the fluorescence has a maximum at about 630 m μ .

When roots which have been grown for a few days in darkness are subsequently transferred into white light the red fluorescence disappears so quickly that after one hour in light it may hardly be noticed. The roots growing in light from the beginning exhibit no red fluorescence at the tips but this appears slowly after transferring into the darkness (in about 24 hours). When investigating the transversal sections of dark growing root tips, red fluorescence is seen only in the cortex.

The present investigations have shown that red fluorescence occurs in the tips of wheat roots grown in darkness in normal solution and also in dark grown root tips of all the species belonging to different plant groups, which have been investigated. The results of this survey are recorded in Table 1.

The dark grown roots of all species which have been examined exhibit distinct or sometimes very distinct red fluorescence. This means that the occurrence of the red fluorescing substance in the root meristem is a common phenomenon.

The distribution of the red fluorescing substance inside the root tips of different species differs slightly when the levels of tissue is under consideration. Usually the strongest fluorescence can be found in the deepest layer of cortex, the weakest in the epidermis (Figure 1 d). In all species, however, it is cytoplasm which contains this substance. In the case of root tips with air filled intercellular spaces the brightest fluorescence seems to be in these spaces. This, however, is due to the light reflection against cell-air surfaces which is evidenced as this intercellular fluorescence disappears after infiltration of the spaces with water.

The roots of wheat and corn were also investigated after 1-NAA treatment. It appears that the red fluorescence is slightly more pronounced after addition of 1-NAA in growth inhibiting concentration to the nutrient solution than without it. The length of zone showing red fluorescence was the same in both cases. It only depends on the size of the root. Only the cell division zone seems to fluoresce. As the growth of cells in 1-NAA-solution is inhibited the concentration of cytoplasm per cell outside the cell division zone increases. One could expect that in 1-NAA treated roots, the length of the red fluorescing zone would be longer if fluorescence depends upon the concentration of the

Table 1. Occurrence of the red fluorescing pigment in dark grown roots.

Species	Source of material ¹	Occurrence		Remarks	
		at root tip	longitudinally transversally		
Pteridophyta:					
<i>Onoclea struthiopteris</i>	1		all tissues	relatively strong fluorescence also very strong yellow fluorescence in peripheral parts in epidermis strong yellow fluores- cence	
<i>Asplenium lucidum</i>	2	"	"		
<i>Pteris cretica</i>	2	"	weak red fluorescence in cen- tral part		
<i>Microlepia strigosa</i>	2	"	strong red fluorescence in cortex		
Gymnospermae:					
<i>Cycas revoluta</i>	2	"	strong in cortex especially in deeper layer, weak in stele, not at all in epidermis	red fluorescence stronger in massive roots	
<i>Tetracclinis articulata</i>	2	"	initial centrum and on the bor- der between cortex and stele		
<i>Sequoia sempervirens</i>	2	"	cortex		
<i>Araucaria araucana</i>	2	"	"		
<i>Pinus silvestris</i> (seedlings)	1	"	"	In some 1-NAA treated roots very strong red fluorescence in epidermis seldom in any other part of meristem or in the mature cells <i>Idem</i>	
Angiospermae:					
<i>Triticum</i> sp.	3	"	cortex		
<i>Triticum</i> sp.	isolated roots	"	"		
<i>Triticum</i> sp.	3	"	cortex		
<i>Zea Mays</i>	1	"	cortex		
<i>Lolium perenne</i>	3	"	"		
<i>Linum usitatissimum</i>	3	"	"		
<i>Helianthus annuus</i>	3	"	"		
<i>Lupinus polyphyllus</i>	3	"	cortex		
<i>Lactuca sativa</i>	3	"	cortex		

¹ Numbers refer to sources as follows:

1: roots from plants growing outdoors

2: roots from plants growing in pots in the greenhouse

3: roots from seedlings growing in the nutrient solution or in vermiculite moistened with nutrient solution.

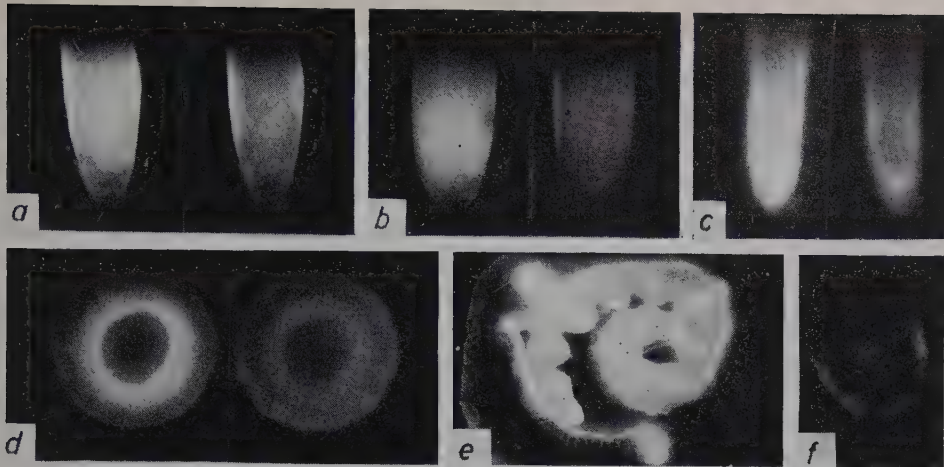


Figure 1. Red (and yellow) fluorescence in *Zea* root and in etiolated wheat leaf. a—c — photographs of intact root tips of *Zea* before and after illumination with strong white light; a, b — normally fluorescing tips, time of illumination (beside exposition) 2 min., c — bright fluorescing tip, time of illumination 4 min. d — photograph of a transversal section from massive root tip of *Zea* 0.3 mm. thick before and after 2 min. illumination. e — transversal section of etiolated wheat leaf 0.3 mm. thick killed before exposition with hot water. f — transversal section from mature part of *Zea* root treated with 1-NAA showing bright red fluorescing epidermis. All pictures were taken under identical conditions. Time of exposure 1 min. During it the object was illuminated with blue light. Magnification the same for all the pictures.

cytoplasm containing the responsible pigment. The lack of dependence of length of fluorescing zone on 1-NAA treatment, however, speaks against this assumption.

Some of the roots of corn as well as of wheat grown in 1-NAA solution give extremely strong fluorescence at the tip or sometimes even in the mature part of the root (Figure 1 c, f). The colour of this bright fluorescence as examined by means of interference filters seems to be the same as in normal tips. The bright fluorescence is usually localized in the epidermis but it may occur in the whole tip including the root cap or in the group of cortical cells in the mature portion of the root. The brightness of this fluorescence is caused in part by superficial localization of cells containing the fluorescing pigments but it is chiefly due to a higher concentration of the pigment. The concentration is so high that one can observe the fluorescence in separate cells under a 100 power water immersion objective. It can then easily be seen that it is localized in cytoplasm though it is still difficult to say if it is distributed uniformly or is in bodies since the light scattering is very high.

Some Properties of the Red Fluorescing Substance

The decomposition of the pigment by light as determined by means of interference filters takes place in both the red and the blue region. Infra-red light (longer than 700 m μ) and green light are quite ineffective. It is difficult, however, to say anything qualitatively about the effectiveness of the different types of light since the intensity of light which passes through the filters is not known. Nevertheless the strong effect of red and blue light deserves to be emphasized. Decomposition by light proceeds rather rapidly as it may be seen from Figure 1 a—d. In strong white light from a Zircon arc lamp nearly all red fluorescing pigment disappears within 4 minutes. With the red portion of this light obtained from an interference filter with transmission at 636 m μ the decomposition takes 10 to 20 minutes. About the same period of time will be necessary with blue light from a 1.5 cm. thick filter of ammonium copper sulphate. This fading of fluorescence in the first minutes of illumination might be due to the reversible rapid change in fluorescence yield analogous to the change in fluorescence of chlorophyll known as the "Kautsky effect" (literature in Rabinowitch 1956) but at any case there is also a true disappearance of fluorescing pigment.

The bright fluorescence which some roots exhibit after 1-NAA treatment is seemingly more stable, but the reason for this may be simply a higher initial concentration of the pigment.

The fluorescence in normal root tips disappears immediately when the tip is squashed. Pulp from freshly ground material also fails to exhibit any red fluorescence. This disappearance of fluorescence as an effect of the squashing can be counteracted by addition of ethyl ether or ascorbic acid or with even better results by addition of both together. Acetone, ethyl-(methyl-) alcohol, KCN, acetic acid, weak alkali have all harmful effects.

The cutting of roots with a sharp razor-blade does not influence the fluorescence of the undamaged cells.

After the roots have been killed with hot water the red fluorescence, though weak and masked by a strong yellow fluorescence resulting from the killing process is not longer sensitive to the squashing of the tip.

However, roots which exhibit abnormally bright fluorescence show no sensitivity to squashing or grinding. Furthermore, these tips have sometimes already died although still brightly fluorescing, which suggests that the process which causes the appearance of the bright red fluorescence also leads to the death of the tissue.

The red fluorescing substance may be fixed in an anatomical sense of the word with 10 % formaldehyde after previous immersion of tips in ether for 10 to 20 minutes. With other fixating agents the fluorescence disappears.

The red fluorescing substance can be extracted but the amounts obtained are small. The best way of extraction seems to be to grind the tips in ether with addition of sand and traces of ascorbic acid at low temperature (0°—4°C) and under dim green light. Extraction with acetone gives poor results with the fresh material.

The crude ether extract from dark grown root tips gives a weak orange fluorescence as seen under a fluorescence microscope or against a dark background in a beam of strong blue light. The same extract from light-treated tips gives only yellow-greenish fluorescence. It is obvious that the fluorescence first mentioned, derives from a mixture of a red and a yellow fluorescing substance. The fluorescing substance in the crude ether extract is rather stable also towards light. Even after 5 hours of illumination with strong red light it was difficult to perceive any difference in the fluorescence. The fluorescence, however, disappears completely during evaporation of the ether extract in vacuum, so that the residue dissolved in the same amount of ether as initially present does not exhibit any red fluorescence but only a yellow one.

When root tips are killed by immersion in hot (90°) water for 15 min. before extraction, the fluorescence is not influenced by the evaporation of ether from the crude extract. In this case acetone may be used for extraction with even slightly better results than with ether.

For a long time the author worked with extracts from fresh material, which was not killed before extraction. All endeavours to purify the fluorescing substances by means of chromatography (cellulose column, saccharose column, paper) were without success. The pigment disappeared completely during the process. However, it is possible to purify the fluorescing substance in extracts from root tips previously killed with hot water. It was found that in this case the pigment is stable when absorbed on paper as well as on saccharose. The only difficulty in obtaining a sufficient amount of the pure pigment is connected with obtaining a large enough amount of root tips.

Identification of the Pigment

As it was difficult to obtain large enough sample of the pigment for identification by absorption spectroscopy, the identification of it was based on its fluorescence. Nevertheless it is worth mentioning that in a crude or partially purified extract from *Zea* root tips (only for this species attempts have been made to establish the absorption spectrum) it is possible to detect weak absorption in the red with a peak about 625 mμ. It was found that the red fluorescing pigment is responsible for this peak. It was not possible to obtain information about the blue light absorption of the pigment in crude extracts

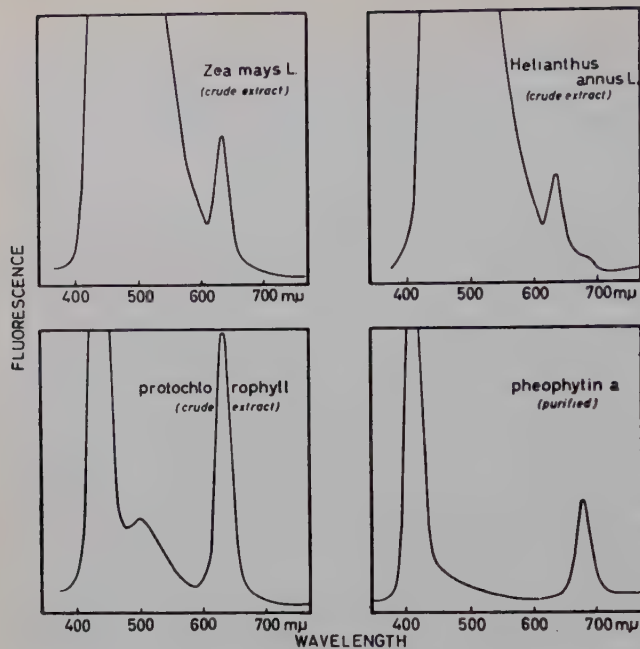


Figure 2. The fluorescence spectra of raw ether extracts from root tips (*Zea* and *Helianthus*) and from etiolated leaves of *Triticum* containing protochlorophyll (activation light 432 mμ) and pheophytin *a* (activation light 410 mμ). Ether solution.

since they contained considerable quantities of carotenoids. The absorption in the red at 625 mμ as well as the previously mentioned data on the fluorescence maximum together with the activation peak at around 430 mμ suggests that the fluorescing pigment is protochlorophyll.

The fluorescence spectrum was obtained with an Aminco-Bowman Fluorospectrophotometer. Reference samples were crude extracts of protochlorophyll *a* obtained from dark grown wheat seedlings according to the method of Virgin (1958), and purified pheophytin *a* obtained in the following way:

50 g. of fresh leaves of *Fagus silvatica* were killed by immersion in hot water for 15 min. and then ground in a mortar together with sand and 50 ml. ether. The slurry was filtered by decantation and after concentration under vacuum diluted with petroleum ether. Repeated evaporation of part of the solvent and dilution with petroleum ether removed the ethyl ether. The petroleum ether concentrate was passed through a saccharose column. The front of the first bluish band was separated mechanically, eluted with petroleum ether and passed twice more through saccharose columns. As only a small quantity of pigment was needed, only the very front of the bluish zone was removed and used in the next step. The absorption spectrum showed that the pigment obtained in this way consisted of pure pheophytin *a*.

Fluorescence spectrometry was applied on the extract from heat-killed *Zea* root tips and on the crude ether extract from fresh root tips of dark grown *Helianthus*. Fluorescence spectrum and activation spectrum were obtained for

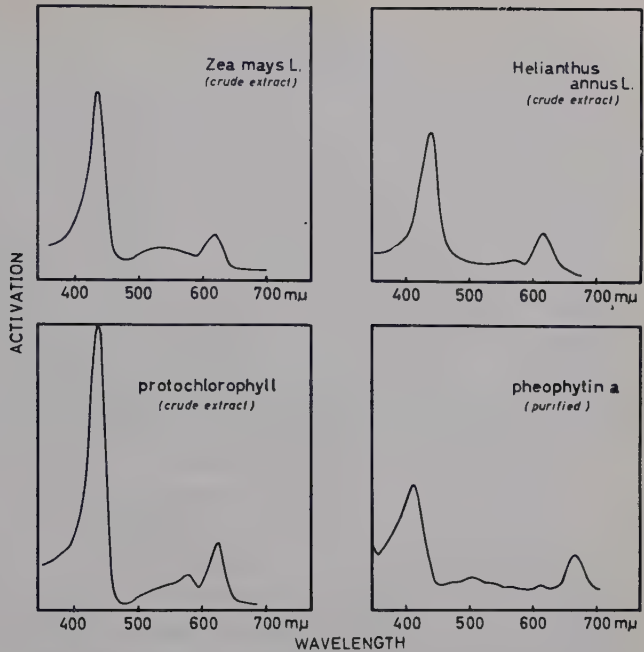


Figure 3. The same as in Figure 2 but activation spectra (the first three for fluorescence at 629 mμ, the fourth one at 672 mμ).

the samples as well as for the references. Calibration of pictures from the oscillograph screen was made in respect to pheophytin *a*. Results are given in Figures 2 and 3.

The positions of the peaks in the spectrum of the red fluorescence is the same for the two samples from *Zea* and from *Helianthus* as well as for the extract from etiolated wheat leaves containing protochlorophyll. By comparison with the pheophytin fluorescence spectrum, the red fluorescence in the mentioned solutions is between 625 mμ and 630 mμ. The wide peak between 400 mμ and 600 mμ, visible in Figures 2 a and b, is due to light scattering which occurs when the wave length of the monochromator analysing the fluorescent light coincides with the wave length of the activation light (sharp peak at 410 in Figure 2 c) and also to yellow fluorescence of impurities.

Activation spectra were determined for red fluorescence at its maximum. They give valuable information about the absorption spectrum within the wave length region showing no fluorescence since only the light which is absorbed can activate fluorescence. The maximum for activation in the investigated samples has the same position as for the protochlorophyll solution and lies at 432 mμ. The peak in the visible red in Figure 3 is partly due to light scattering.

On the basis of the determination of the fluorescence there seems to be little doubt that the pigment in question is protochlorophyll a.

It is possible to get some information on the concentration of the red fluorescing pigment in the root tips by comparing the intensity of its fluorescence with the fluorescence of etiolated wheat leaves in which the concentration of protochlorophyll is known. For this purpose dark-grown seven-days old leaves of wheat were killed by immersion in hot water for 10 minutes (to prevent the transformation to chlorophyll) and transversal sections of about 0.3 mm. in thickness were prepared. Similar sections were prepared from *Zea* root tips. Pictures of the sections taken under identical conditions are given in Figure 1. The red fluorescence in normal root tips of *Zea* is of less intensity than in etiolated leaves of *Triticum*. The protochlorophyll concentration in wheat leaves is 0.005 mg./g. according to Virgin (1958).

Discussion

Considering the common occurrence of protochlorophyll in root tips as it is shown in the present work it is surprising that it has not been noticed earlier. There are, however, a few reasons for this:

1) In microscopic investigation of fluorescence UV light is usually used for activation; 2) As the concentration of pigment is very low it may be difficult to see the fluorescence when the intensity of light, suitable for activation, is weak; 3) The pigment is rapidly destroyed in light and without adequate precautions it may disappear before the operation is made; 4) The pigment occurs in detectable amount only in meristematic parts of roots; 5) The pigment is strongly destroyed by normal methods of extraction.

The chief question now is: why and how is the protochlorophyll formed in root tips?

Protochlorophyll occurs in small amounts in etiolated parts of shoots where it is transformed into chlorophyll a by a photochemical reaction (Smith 1948, Virgin 1958). It also occurs in the seed coats of Cucurbitaceae. No transformation to chlorophyll occurs in the seed coats, however, nor can isolated protochlorophyll be converted. A binding to protein (Smith and Young 1956) is necessary in the transformation.

The function of protochlorophyll in seed coats is quite obscure. There are some evidence that protochlorophyll as well as chlorophyll may act as a sensitizer of oxidoreduction in vitro and itself can undergo both chemical and photochemical reversible reduction (for literature see Rabinowitch 1956, chapters 35 and 37 B).

It is not known if the protochlorophyll in the root tips can be converted to

chlorophyll by illumination. Fluoroscopy of investigated samples of root tip extracts does not reveal any peak at 680 m μ as would be expected if chlorophyll were present. But on the strength of this fact alone an assumption that transformation does not occur in root tips is scarcely valid. It is possible that such a transformation exists in light but that chlorophyll is not protected against destruction such as occurs in albino mutant plants (Koski *et al.* 1951). On the other hand it is worth mentioning, however, that in root tips there is some factor present which specifically causes destruction of protochlorophyll even in green light (Hejnowicz 1958). It is not known if there is any relation between the protochlorophyll in the root tip and the chlorophyll formed in mature part of the root (Burström and Hejnowicz 1958).

It is possible that protochlorophyll exists in all parts of the plant including the mature part of the root and that it is one link in some larger basic metabolic chain. The sporadic occurrence of bright red fluorescence in tissues where it normally does not occur suggests such a possibility. It should be remembered that protochlorophyll is stable in these parts when the tissue is destroyed while in normal root tips, on the contrary, it immediately disappears. It means that the transformation path is blocked there. This blocking would be responsible for the high storing of protochlorophyll.

The well-known action spectra for reversible red-far red interactions which control many processes (photoperiodism, germination of some seeds, etiolation, chlorophyll formation, endogenous rhythm) indicate that they all have a common photoreceptor and that this is to find within the porphyrin class to which protochlorophyll belongs. It is possible that this general photoreceptor occurs in the same metabolic chain as protochlorophyll, perhaps very close to it.

Protochlorophyll was recently shown by Virgin (1958) to be directly involved in one process belonging to the "red-infra red" class of photoreaction; namely, in photocontrolled acceleration of chlorophyll formation.

If protochlorophyll plays an important role in the root meristem the meristem ought to undergo some change as a result of the illumination causing protochlorophyll destruction. Investigations on the influence of light on root growth indicate that there is a distinct inhibition of growth with an action spectrum similar to the spectrum of protochlorophyll destruction (Kohlbecker 1957, Burström and Hejnowicz, unpubl.).

It is possible that protochlorophyll controls some redox processes and is especially important in root growth under conditions where oxygen seems to be at a minimum. This possibility is supported as by the findings of Guminski *et al.* (1955) that roots possess a special (beside normal) respiration mechanism in which other H-acceptors like humic acid are substituted for oxygen. Some observations by the present author support this hypothesis:

wheat roots die in nutrient solution "aerated" with nitrogen in light but not in darkness. Additional investigations, however, are needed before any accurate conclusion about the role of photochlorophyll in root tip can be drawn.

Summary

Protochlorophyll seems to be generally present in root tips. It has been shown to occur in all species investigated belonging to different plant classes ranging from ferns to angiosperms. It is rapidly destroyed in red and in blue light. Protochlorophyll occurs also sporadically in some cells of 1-NAA treated roots (in meristem or in mature part) in abnormally high concentrations. Its significance is discussed.

The author wishes to express his sincere gratitude to Professor H. Burström and Dr. H. Virgin for help and suggestion during the course of the investigation. He is also much indebted to Dr. P. A. Carlsson and Mr. T. Magnusson (Farmacological Institute, Lund) for carrying on fluoroscopy measurements, to Dr. A. Persson for plant material and to Dr. R. Cleland for correcting the English text.

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Iron Uptake in Different Plant Species as a Function of the pH Value of the Nutrient Solution

By

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1. Introduction

It is a well-known fact that the hydrogen ion concentration of the soil is a significant factor governing the distribution of plants in nature: each plant species is found only in soil having a pH value within a range characteristic of the species in question (Olsen 1923).

Certain species (acid soil plants) occur only in soil of a pH between 3.6 and 5.0. Other species (basic soil plants) grow only in soil of a pH between 6.0 and 8.0. Comparatively few species are definite acid soil plants, while numerous species belong to the group of basic soil plants. Furthermore there are some species that are found in strongly acid as well as slightly acid, neutral, or slightly alkaline soil. Cultivation experiments with several of these species (*Spergula arvensis*, *Rumex acetosella*, *Convallaria majalis*, *Majanthemum bifolium*) have shown that they grow equally well in soils with pH values covering the range from 4.0 to 8.0 (Olsen 1936).

Water culture experiments with typical acid soil plants and basic soil plants have shown that the plants respond to the pH value of the solution in much the same way as to the pH of the soil. In solutions of pH values between 4.0 and 5.0, *Deschampsia flexuosa* shows optimum growth. When the pH increases growth slows down, and in solutions within the pH range 6.0 to 8.0 chlorosis and pronounced inhibition of growth are observed (Olsen 1938). *Sinapis alba* shows optimum growth in solutions of pH 6.0 to 8.0. In the pH

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range from 4.0 to 6.0 growth is inhibited, and in solutions of pH 4.0 or lower this plant species cannot develop at all (Olsen 1935). In contradistinction to these two species, a plant like rye (*Secale cereale*) proves indifferent to the pH value of the solution in so far as it shows optimum growth in solutions covering the entire range from 4.0 to 8.0. It has been demonstrated that hydrogen ions are only directly harmful to rye plants at pH values of 3.5 or lower, while direct harmful effects of hydroxyl ions occur only at pH values around 10.5 or higher (Olsen 1953).

This raises the following questions: Is the inhibition of growth observed in *Deschampsia flexuosa* when the pH of the solution is increased from 6.0 to 8.0 due to the rise in hydroxyl ion concentration? And conversely, is the increasing growth inhibition observed when *Sinapis alba* is cultivated in solutions of pH values decreasing from 6.0 to 4.0 caused by the increasing hydrogen ion concentration? Neither assumption seems probable. The fact that it has not until now proved possible to make either acid soil plants or basic soil plants develop to the optimum extent in solutions of arbitrary pH values within the range from 4.0 to 8.0, is hardly due to the pH in itself, but to other factors which, under the experimental conditions hitherto employed, vary concurrently with the pH value. In soil these factors are inextricably bound to the pH value and cannot be altered independently, but in solution culture this might be possible. That it actually is so will be seen from the experiments described in the following.

2. Methods

As nutrient solution a four-salt solution was used; it contained three cations and three anions in the concentrations indicated in Table 1.

Glass-distilled water was used. In addition to the four salts listed in Table 1, the solution contained trace elements. The amounts of their compounds per liter were 1 mg $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ — 0.8 mg H_3BO_3 — 0.2 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ — 0.05 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ — 0.05 mg $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$. Iron was added either as ferric sulfate or as iron versenate; in either case the concentration was 0.5 mg Fe per l.

The iron versenate solution was prepared by dissolution of 2.61 g Complexone II (ethylenediamine-tetra-acetic acid, Siegfried) in 26.8 ml 1 M KOH, addition of 2.49 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, dilution to 100 ml, and aeration for twenty-four hours.

Each nutrient solution was adjusted to the desired pH value by addition of sulfuric acid or potassium hydroxide. The plants were cultured in glass cylinders 20 cm high by 19 cm wide, each containing five liters of nutrient solution. The vessel was covered with a galvanized iron plate with four holes. A plant was placed in each of these holes, supported by a flat, perforated cork. The solution was aerated with a continuous stream of air throughout the experimental period.

The contents of iron, calcium, magnesium, and potassium in the plants were determined by methods previously described (Olsen 1934, 1939, 1942).

Table 1. *Composition of the nutrient solution.*

Per liter	m.eq. per liter					
	K	Ca	Mg	NO ₃	H ₂ PO ₄	SO ₄
0.354 g Ca(NO ₃) ₂ , 4H ₂ O ..	—	3.0	—	3.0	—	—
0.370 g MgSO ₄ , 7H ₂ O	—	—	3.0	—	—	3.0
0.270 g KNO ₃	2.67	—	—	2.67	—	—
0.046 g KH ₂ PO ₄	0.33	—	—	—	0.33	—
Total	3.0	3.0	3.0	5.67	0.33	3.0

3. Experiments with *Deschampsia flexuosa*

Deschampsia flexuosa (bent grass) grows on waste land and in forests on raw humus. It occurs frequently on soil having pH values from 3.5 to 5.0, but not above 5.0 (Olsen 1923). If it is sown on soil of pH in the range 4.0 to 8.0 it develops most vigorously at pH values between 4.0 and 5.0, but growth declines rapidly above pH 5. In soil of pH values from 6.0 to 8.0 the germinating plants soon become chlorotic and then do not develop further (Olsen 1923, 1936, 1938 II). In solution cultures the plants have behaved in the same way in all experiments previously reported: Already in solution of pH 6.0 definite chlorosis is observed, and at pH 7.0 no growth occurs at all (Olsen 1923, 1938 II). It is certain that the chlorosis is due to iron deficiency. In previous investigations into chlorosis in corn plants, conspicuous in solution of pH 7.0, it was found (Olsen 1935) that chlorosis could be avoided when iron was added to the neutral solution as ferric citrate, in which the iron is bound in complex form. Later (Olsen 1938 b) a number of *D. flexuosa* experiments were performed in which the composition of the solution was varied in several ways. Although ferric citrate was used as source of iron, it did not prove possible to make the plants develop in solutions of pH 7.0 or 8.0.

First in 1957 new experiments were undertaken with *D. flexuosa*. In place of ferric citrate, which is rather unstable in solution, ferric versenate was used. This compound contains its iron in very stable chelate binding; in numerous cases it has proved an excellent iron source for plants in solution culture. It has been used by the present author as iron source for *Helodea canadensis* (Olsen 1954).

Small plants of *D. flexuosa* were collected from their natural habitat and precultured in the above solution at pH 4.0 and with ferric sulfate as iron source. After about one month the plants had formed vigorous new roots; now they were transferred to the vessels used for the growth experiments proper. Their initial weight of dry matter was ca. 3 g per vessel holding four plants. The plants were cultured in solutions of pH 4.0 and 7.0, respectively.

Table 2. *Deschampsia flexuosa* in nutrient solution.

pH of solution	Iron source	Increase in dry matter, ¹ g	mg per g leaf dry matter			
			Fe	Ca	Mg	K
4.0	Ferric sulfate	33.4	0.13	3.1	1.17	22.1
	Iron versenate	32.1	0.15	3.5	1.30	24.2
7.0	Ferric sulfate	3.6	0.10	12.4	4.88	55.0
	Iron versenate	31.6	0.16	4.1	1.93	22.3

¹ The increase in dry matter is taken as the weight of dry matter of four plants at the end of the experiment minus 3.0 g (the dry matter weight of four plants at the beginning of the experiment).

At each pH value ferric sulfate was used as iron source in some experiments, iron versenate in others. The experimental period was a little more than four months (January 3 to May 9, 1957). The cultures stood in a greenhouse where the temperature was maintained between 15 and 20°C. During the first three months they were illuminated by means of fluorescent lamps. During the experimental period the solutions were changed eight times. Their pH values were checked daily; whenever the pH of a solution differed more than 0.2 from its specified value, it was corrected by dropwise addition of 1 *M* sulfuric acid or potassium hydroxide.

The plants having iron versenate as source of iron grew vigorously with a considerable increase in dry matter. This was true in solution of pH 7.0 as well as at pH 4.0 (Table 2); in both solutions the leaves were a deep green.

The plants grown on ferric sulfate as iron source likewise developed vigorously at pH 4.0, but in solution of pH 7.0 the plants soon grew chlorotic (Figure 1), and after termination of the experiment the increase in dry matter proved to be only one-ninth of that of the plants cultured at the same pH, but with iron versenate as source of iron (Table 2).

Table 2 further shows that the relative iron content is least in the leaves of the chlorotic plants. The chlorosis is not only caused by the fact that the plants cannot take up sufficient quantities of iron from the solution, but also by difficulties in internal translocation, the iron being precipitated in the veins so that it is unable to pass into the mesophyll. This has been shown in previous experiments with corn plants (Olsen 1935) and has been confirmed by Rediske and Biddulph (1953) in experiments on *Phaseolus vulgaris*.

Furthermore, Table 2 shows that the contents of calcium, magnesium, and potassium in the leaves of the chlorotic plants are almost three times those in the leaves of the normal, green plants. Previous experiments (Olsen 1935)

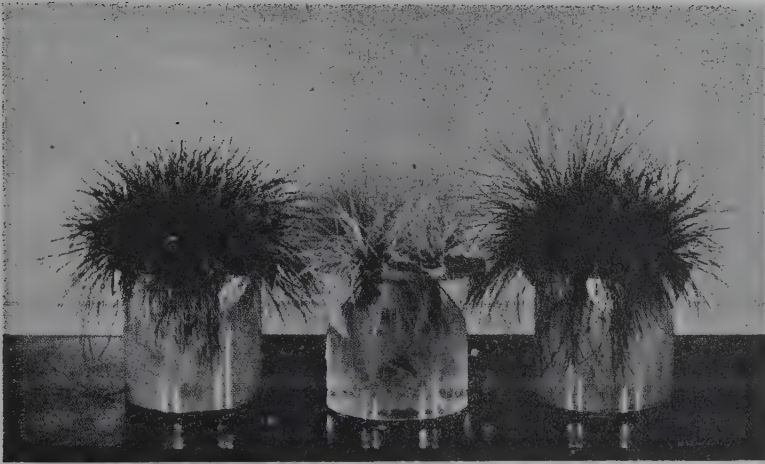


Figure 1. *Deschampsia flexuosa* in nutrient solution. The plants to the left had developed in solution of pH 4.0 with ferric sulfate as iron source. The chlorotic plants in the middle had developed in solution of pH 7.0 containing ferric sulfate. The plants to the right had developed in solution of pH 7.0, but with iron versenate as source of iron.

Table 3. Contents of Fe, Ca, Mg, and K in leaves of *Deschampsia flexuosa* taken from two different localities on the Danish island of Zealand (Sjælland); mg per g dry matter.

Locality	Fe	Ca	Mg	K
Gribskov	0.13	1.12	1.01	20.4
Gyldenløveshøj	0.14	1.24	1.13	15.3

showed chlorotic corn plants to contain far more calcium, magnesium, and potassium than normal, green corn plants. This must presumably be explained by the assumption that, whereas the dry matter production of the chlorotic plants soon slows down, the uptake of salts from the solution continues so that the inorganic constituents become associated with a smaller quantity of dry matter than in the normal, green plants.

Finally, Table 2 shows that, apart from the chlorotic plants, the calcium content of the leaves of the experimental plants is very low, viz. ca. 3.5 mg Ca per g dry matter. When *Cannabis sativa* was grown in the same solution, in which calcium, magnesium, and potassium were present in equivalent concentrations, its leaves contained 48.0 mg Ca per g dry matter, or about fourteen times as much as *D. flexuosa*. The low calcium uptake is characteristic of *D. flexuosa*. It is seen from Table 3 that the calcium content of the leaves is still lower in the natural habitat of this plant, while the contents of potassium and iron are not very different from those in the experimental plants. The extremely low calcium content in the leaves from the natural localities is of course a consequence of the very low calcium concentration of the strongly acid raw humus.

If the Tables 2 and 3 are recalculated as equivalents it will be seen that the Ca/Mg ratio is ca. 1.5 in the leaves of the experimental plants, but only 0.67 in the leaves of the plants from the natural habitat. In other words, there is relatively more magnesium than calcium in the plants from the raw humus, and this must be due to the fact that this strongly acid soil contains relatively more accessible magnesium than calcium.

The main result of the experiments is that *D. flexuosa* can attain optimum growth in solution culture throughout the pH range 4.0 to 8.0 when iron is added as iron versenate. When ferric sulfate is the iron source the plants grow chlorotic in solutions of pH 6.0 to 8.0, and the production of dry matter ceases.

Similar conditions prevail in soil, and the reason why *D. flexuosa* cannot grow in soil at a pH above 6.0 is the fact that it is unable to absorb a sufficient quantity of iron.



Figure 2. *Hemp* in soils of different pH values. The soils in the left and middle pots were forest soils, that in the right pot garden soil. To all the pots identical quantities of potassium nitrate and potassium dihydrogen phosphate were added. The plants were transplanted as seedlings.

4. Experiments with *Cannabis sativa* and *Secale cereale*

Figure 2 shows hemp growing in soils of pH values 4.0, 5.0, and 7.0, respectively. It will be seen that the optimal pH for growth of hemp is in the neighbourhood of 7. In soil of pH 4 the plant is almost unable to grow, and in soil of pH 5 growth is considerably retarded.

In some studies of competitive ion absorption commenced in 1955, hemp (*Cannabis sativa* var. *gigantea*) and rye were used as experimental plants. The latter is a typical pH-indifferent species. Originally it was not intended to study the responses of the two species to the pH values of the solutions, the author having no notion at the time that hemp was a typical "basic soil plant". The reason why hemp was introduced was the known fact that, in contrast to rye, hemp had an extraordinarily high rate of calcium absorption.

Both plant species were grown in solutions containing six different concentrations of calcium ranging from 0 to 30 m.eq. per l. The solutions had the composition given in Table 1 except for the calcium nitrate. Iron was added as ferric sulfate; the other trace elements were unchanged. The pH was kept constant at 5.0 since this value had proved favorable in experiments with rye. This soon proved not to be true in the case of hemp, and just this fact made



m.eq. Ca/l	0	0.5	1.0	3.0	10.0	30.0
Dry matter, g	1.4	4.1	4.4	4.5	4.4	3.8

Figure 3. *Rye in solutions of increasing calcium concentration. pH 5.0 throughout.*

it possible to obtain information concerning the peculiar behaviour of hemp in acid solutions.

When the experiments had lasted four weeks, the plants were photographed, dried, and weighed, and their contents of calcium, magnesium, and potassium were determined.

Figure 3 shows the rye plants. Below each plant the calcium content of its nutrient solution and the dry weight of the plant at the conclusion of the experiment are stated. It is seen that rye can develop to a certain extent even in a solution devoid of calcium. To be sure, the plants grown in such a solution were not free of calcium; four plants contained a total of 0.85 mg Ca, which had been present in the seedlings before they were placed in the solution. Even in a solution containing only 0.5 m.eq. Ca per l the dry weight approached the optimum. On the other hand, the highest calcium concentration, viz. 30 m.eq. per l, decreased the dry weight somewhat.

Figure 4 shows hemp in the same solutions. It is seen that the dry weight increased throughout with increasing calcium concentration in the solution. In calcium-free solution the plants did not develop at all. In the solution containing 0.5 m.eq. Ca per l, where the dry weight of rye approached the optimum, the dry weight of hemp was only one-fourth of that attained by the plants growing at the highest calcium concentration. This experiment might lead one to the conclusion that hemp required large amounts of calcium for optimum development, which would be in accord with the views of the botanists of the nineteenth century, who thought that plants growing preferentially on calcareous soil required large quantities of calcium (Schnitzlein and Frick-

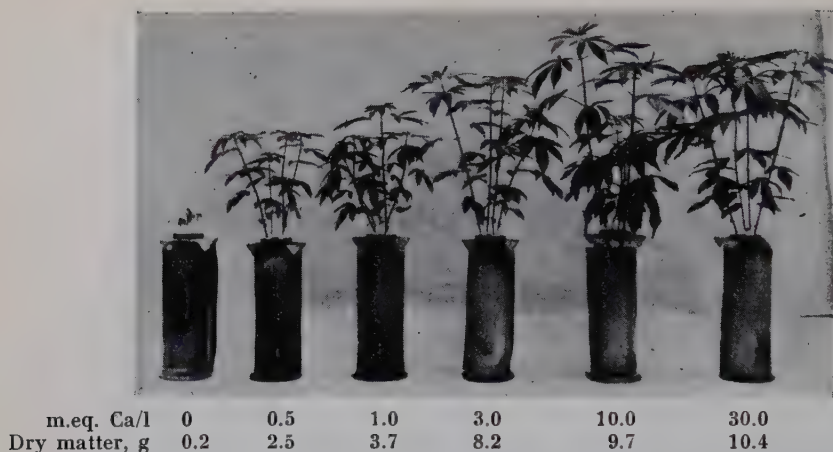


Figure 4. *Hemp* in solutions of increasing calcium concentration. pH 5.0 throughout.

hinger 1848). However, the situation is not so simple. The well-known symptoms of calcium deficiency, such as dying of growing points, were not observed in the feebly developed hemp plants, so that there was no reason to believe that the growth inhibition in the solutions of low calcium content was due to shortage of calcium. More probably it was caused by a toxic effect of one or more cations of which too much was absorbed. In a solution of high calcium ion concentration the absorption of the other cations present is depressed as a result of competition, and the toxic effect is alleviated.

The results of competition between cations in hemp and rye are shown in Figure 5, which shows the contents of calcium, magnesium, and potassium in the plants at the termination of the experiment. When the calcium concentration in the solution was increased, both plant species absorbed increasing quantities of calcium. At the same time the uptakes of magnesium and of potassium went down, particularly that of magnesium, since competition is always more pronounced between more closely related ions, in the present case the two divalent cations. Furthermore, the figure shows that there was a characteristic difference between hemp and rye in that hemp absorbed three to four times as much calcium as did rye, and also more magnesium. However, the Mg/Ca ratio was higher in rye. There was no marked difference in the quantities of potassium taken up.

Now the question is: Which of the cations present in the solution inhibits the growth of hemp when the calcium ion concentration is low? Some experiments that will not be described in detail here showed that the magnesium ion concentration could be increased considerably relative to the calcium ion

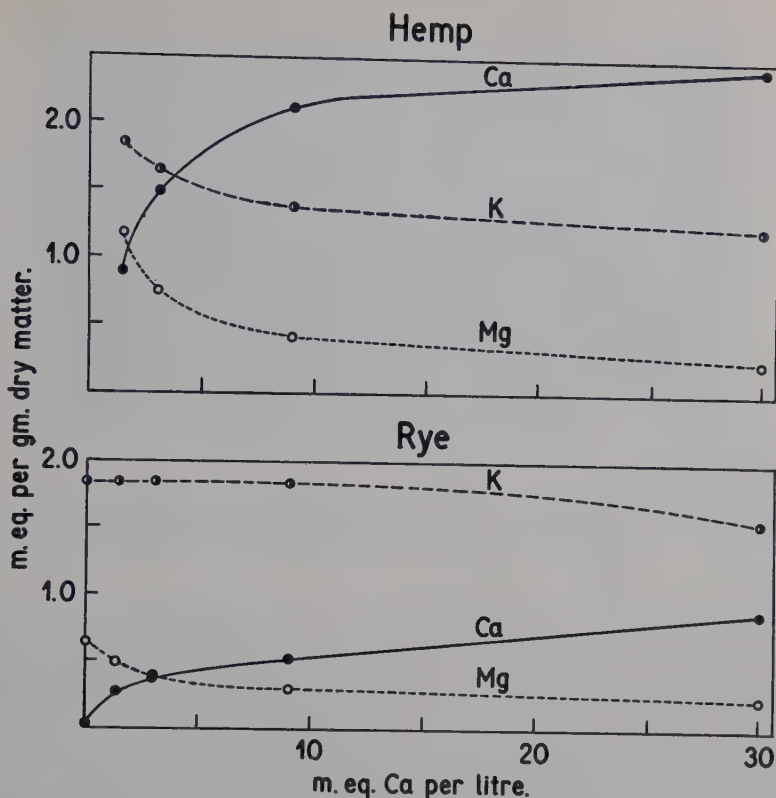


Figure 5. The calcium, magnesium, and potassium contents of hemp and rye grown in solutions of increasing calcium concentrations. pH 5.0 throughout. Roots, stems, and leaves were analyzed separately, and the total content of each ion in four plants was calculated.

concentration without affecting the development of the hemp plants. Hence the toxic ion could not be the magnesium ion. Attention was now directed to the trace elements. It was found that a considerable increase of the concentrations of manganous and zinc ions in a solution containing 10 m.eq. Ca per l caused no growth inhibition, while a considerable decrease of these ions in a solution containing 1 m.eq. Ca per l did not abolish the inhibition. So neither the zinc nor the manganous ion was toxic. This made it probable that the toxic effect was due to the ferric ions present.

An increase in the concentration of ferric ions, with a view to enhancing the growth inhibition, could only be effected by lowering the pH of the solution since the ferric ion concentration attainable was limited by the solubility of ferric hydroxide. In the above experiments the pH had been 5.0; it was now lowered to 4.0.



Figure 6. *Hemp* in solutions of pH 4.0 with ferric sulfate as iron source. The concentrations of calcium and magnesium in the solutions were varied.

Figure 6 shows the result of an experiment with hemp in solution of pH 4.0 containing ferric sulfate as source of iron; the contents of calcium and magnesium were varied. It is seen that when the calcium ion concentration was sufficiently high, viz. 10 m.eq. per l, hemp developed quite normally even in solution of pH 4.0. If the calcium concentration was decreased to 1 m.eq. per l, growth inhibition occurred, and if both the magnesium and the calcium concentrations were reduced to 1 m.eq. per l, the inhibition was enhanced; this may be ascribed to reduction of the competitive effect of the magnesium ions. The plants exhibited distinct symptoms of intoxication.

The intoxicated plants had small, deep green leaves containing as much as 1.3 mg iron per g dry matter, which is about four times as much as found in leaves of normal plants. The roots were short and ramified in a way that was reminiscent of corals; they had no root hair. Growth soon came to a standstill, no fresh leaves or roots developing. Hemp seedlings planted into soil of pH 4.0 soon showed the same symptoms: deep green leaves of a high iron content, short, coral-shaped roots, and soon cessation of growth.

If iron was omitted from the solutions, the intoxication symptoms did not occur: The plants developed normally forming long, white roots. However, growth soon ceased because of iron deficiency.

The experiment of Figure 6 did not prove directly that the intoxication of the plants in the solution containing 1 m.eq. Ca per l was due to an excessive uptake of ferric ions. The toxic effect might be due to the hydrogen ions, and a high concentration of calcium ions might have an antagonistic effect suf-



Figure 7. Hemp in solutions of pH 4.0 and 7.0 with ferric sulfate or iron versenate as iron source. Concentrations of calcium and magnesium both 1 m.eq. per l throughout.

ficient to counterbalance the toxicity of the hydrogen ions. But the following experiment showed that this was not the correct explanation. It proved possible to obtain normal, vigorous plants in solution of pH 4.0 even when the concentrations of calcium and magnesium were only 1 m.eq. per l. This was effected simply by replacing the ferric sulfate in the solution by iron versenate so that the concentration of free ferric ions became extremely low. Figure 7 shows the result of an experiment in which all the plants had grown in solutions containing 1 m.eq. Ca, 1 m.eq. Mg, and 0.5 mg Fe per l. In solution of pH 4.0 the plants were intoxicated when the iron source was ferric sulfate, but when it was iron versenate normal, vigorous plants developed. This experiment clearly showed that the intoxication was due to the ferric ions. In solution of pH 7.0 the plants that had ferric sulfate as iron source were feebly developed owing to iron deficiency caused by the very low solubility of ferric iron in solution of pH 7.0. The plants that had been given iron versenate were vigorous and had developed normally.

Thus, when ferric sulfate was the iron source, the plants were intoxicated in solution at pH 4.0 because they absorbed too much iron, but in solution of pH 7.0 growth was inhibited because an insufficient amount of iron was available to the plants.

With iron versenate as source of iron the plants developed normally and grew just as well at pH 4.0 as at pH 7.0 even when the concentrations of calcium and magnesium were low.

When, as in the experiment illustrated in Figure 8, the concentrations of



Figure 8. *Hemp* in solutions of pH 4.0 and 7.0 with ferric sulfate or iron versenate as iron source. Concentration of calcium 10 m.eq. per l, of magnesium 3 m.eq. per l.

calcium and magnesium were high, viz. 10 m.eq. Ca and 3 m.eq. Mg per l, it proved immaterial whether iron was added to the solution as ferric sulfate or as iron versenate as long as the pH was 4.0. In both cases vigorous, well-developed plants were obtained. In solution of pH 7.0 well-developed plants were obtained only with iron versenate as source of iron, just as in the experiment of Figure 7. When the iron source was ferric sulfate the plants suffered from iron deficiency, and even more so than in the previous experiment, because competition was keener when the calcium and magnesium concentrations had been raised.

It might seem strange that when hemp is grown in solution of pH 7.0 with ferric sulfate as source of iron, it suffers from iron deficiency, which it usually does not when growing in soil of pH 7.0. The deficiency is due to the fact that more than 99 per cent of the iron added is present as ferric hydroxide forming a sediment at the bottom of the vessel containing the nutrient solution. When the plants have absorbed the iron remaining in solution, the ferric hydroxide dissolves only very slowly. However, previous experiments with corn (*Zea mays*) (Olsen 1938) have shown that it is possible to provide a sufficient quantity of iron in solution at pH 7.0 if the amount of precipitate is increased and if it is kept in suspension by very vigorous stirring.

In a final experiment (Figure 9), hemp was grown in solution of pH 4.0 and with low calcium and magnesium concentrations. As expected, the experiment shows that iron intoxication cannot be prevented by addition of iron versenate when the solution contains ferric ions.



Figure 9. *Hemp* in solution of pH 4.0. Calcium and magnesium concentrations 1 m.eq. per l. The plants to the left had ferric sulfate as iron source, those to the right iron versenate, those in the middle both ferric sulfate and iron versenate.

5. Discussion

The experiments show that *Deschampsia flexuosa* and hemp can attain optimum growth in solution throughout the pH range 4.0 to 8.0 when iron is added as iron versenate.

If ferric sulfate is employed as source of iron *D. flexuosa* cannot develop in solutions of pH higher than 6.0; the plants grow chlorotic and the production of dry matter ceases because of iron deficiency. In solutions of low calcium concentration and with ferric sulfate as source of iron, hemp shows increasing growth inhibition due to iron intoxication when the pH values of the solutions decrease from 6.0 to 4.0. In this pH range the ferric ion concentration of the solution increases greatly when the pH falls as shown by Figure 10. It will be seen that the nutrient solution used in the experiments (Table 1) can only contain 0.002 mg ferric ion per l in stable solution at pH values above 6.0. However, when the pH decreases from 6.0 to 4.0, the solubility of ferric iron increases considerably, and this causes the rate of iron absorption to rise as long as the concentration is below 0.003 m.eq. ($=0.056 \text{ mg Fe}^{+++}$ per l (Olsen 1950).

With ferric sulfate as iron source, hemp can develop quite normally even in solution of pH 4.0 if the calcium concentration is raised to 10 m.eq. per l so that the Fe/Ca ratio is diminished. The competition between the calcium and ferric ions causes the rate of iron absorption to decrease so far that intoxication does not occur.

It is difficult to say anything about the mechanism of iron intoxication. As mentioned above the leaves of the intoxicated plants contain as much as 1.3 mg Fe per g dry matter, which is about four times as much as in leaves

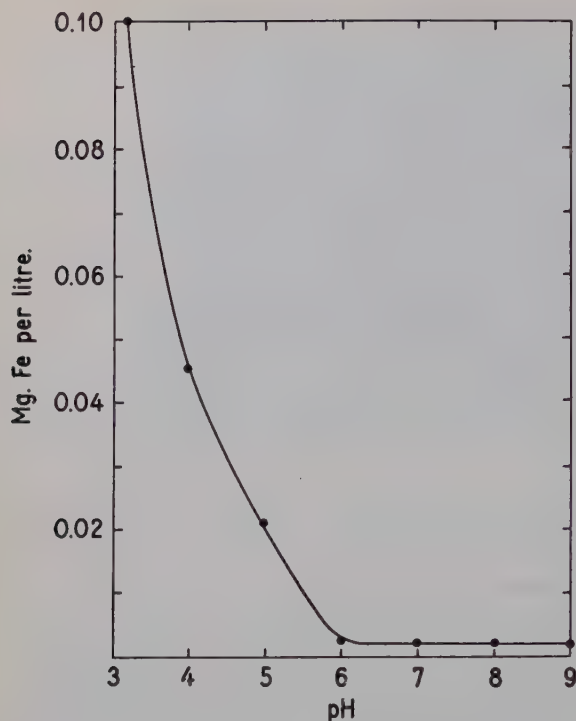


Figure 10. Ferric ion concentrations (as mg Fe per l) in nutrient solutions as a function of the pH value.

of normal plants. In roots of intoxicated plants iron contents as high as 8.0 mg per g dry matter have been found. However, these data are not a true expression of the real iron contents of the roots because an appreciable proportion of the iron is present as ferric hydroxide adhering to the surface. In the intoxicated plants water absorption was inhibited. This was shown by the fact that their leaves grew flaccid and drooping when the temperature of the greenhouse rose; this did not occur in normal plants.

Sinapis alba has previously been used by the author as an example of a basic soil plant. In a few experiments done in 1956 it behaved just like hemp. In solution of pH 4.0, with a low calcium content, and with ferric sulfate as iron source, the *S. alba* seedlings developed into small, deep green plants, which soon ceased to grow because of iron intoxication. The intoxication could be avoided either by the use of iron versenate as source of iron or by increasing the calcium concentration of the solution to 10 m.eq. per l. In both cases normal, well-developed plants were obtained.

When rye is grown in solution of pH 4.0 and with a low calcium content, normal, well-developed plants appear when the iron source is ferric sulfate, but still the production of dry matter is increased somewhat if iron versenate

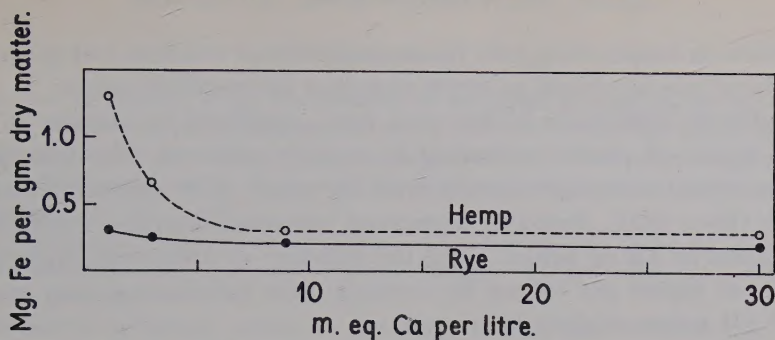


Figure 11. The iron contents of leaves of hemp and rye grown in solutions of four different calcium concentrations. The pH value was 4.0 throughout.

is used in place of ferric sulfate. This shows that rye is not quite unsusceptible to iron intoxication although its susceptibility is much less than that of hemp.

The difference is presumably a reflection of the difference in the rates at which the two plant species absorb ferric ions. Rye absorbs slowly, hemp considerably more rapidly.

This is seen from Figure 11, which shows that leaves of hemp that had grown in solution of pH 4.0 and a low calcium content, viz. 1 m.eq. per l, contained four times as much iron as did leaves of rye that had grown in the same solution. The iron content in leaves of *Deschampsia flexuosa* suggests that this plant species has a still lower rate of iron absorption than has rye. It is seen from Tables 2 and 3 that both the experimental plants and the plants that had grown in natural localities had a lower iron content in their leaves than had rye. The plants from Gribskov had grown on soil of pH 4.0 having a very low content of calcium ions, which may be seen from the extremely low calcium content of the leaves, viz. 1.12 mg Ca per g dry matter. In spite of this the iron content of the leaves is no higher than in the experimental plants, which had grown in solution containing 3 m.eq. Ca per l. This suggests that the rate of iron absorption is not, or only slightly, increased even by a great reduction of the calcium concentration in the solution (this experiment has not been done), and that consequently *D. flexuosa* is not susceptible to iron intoxication.

We have seen that the rate of iron absorption is highest in hemp, lower in rye, and presumably still lower in *D. flexuosa*. The rates of absorption of the divalent ions calcium and magnesium decrease in the same sequence: Hemp has the highest rates of absorption of these ions, *D. flexuosa* the lowest.

Thus the fact that "basic soil plants" show increasing growth inhibition with increasing hydrogen ion concentration in the soil is not directly due to the increase in hydrogen ion concentration, but to the increased solubility of

iron, which, in conjunction with the low contents of calcium and magnesium, causes the plants to absorb so much iron that intoxication occurs.

Undoubtedly aluminum intoxication may contribute to making it impossible for basic soil plants to develop in strongly acid soil. The symptoms of aluminum intoxication have been known for many years (Hartwell and Pember 1918, Olsen 1923). Probably aluminum intoxication makes itself felt only at pH values of 4.0 or below, since the solubility of aluminum hydroxide is negligible at higher pH values. In contrast, iron intoxication may occur already at pH values slightly below 6.0.

Summary

The acid soil plant *Deschampsia flexuosa* and the basic soil plants *Cannabis sativa* and *Sinapis alba* can attain optimum development in solution culture throughout the pH range from 4.0 to 8.0 when iron is added as iron versenate.

With ferric sulfate as source of iron *D. flexuosa* cannot develop in solutions of pH above 6.0; the plants become chlorotic and the production of dry matter comes to a standstill because of iron deficiency. The same is true in soil of pH higher than 6.0.

When hemp (*C. sativa*) and mustard (*S. alba*) are cultured in solutions low in calcium and with ferric sulfate as iron source, increasing growth inhibition due to iron intoxication is observed when the pH value of the solution decreases from 6.0 to 4.0. This is due to the fact that in this pH range the ferric ion concentration in the solution increases greatly when the pH is lowered. The same is true in soil. Thus the fact that hemp and mustard do not thrive in soil of pH values appreciably lower than 6.0 is not due directly to the hydrogen ion concentration, but to intoxication caused by an excessive rate of iron absorption.

Even with ferric sulfate as iron source, hemp and mustard can develop quite normally in solution of pH 4.0 provided the calcium ion concentration of the solution is high. This is due to the competitive action of the calcium ions resulting in a sufficient lowering of the rate of iron absorption to preclude intoxication. This antagonistic situation does not occur in Nature since soil of low pH and high calcium concentration does not exist.

In *D. flexuosa* and rye (*Secale cereale*) iron intoxication does not occur, as it does in hemp, when the plants grow in soil of pH 4.0 or in solution of this pH and low in calcium. The difference between the three plant species in this respect is probably a reflection of the difference in rate of absorption of ferric ions. *D. flexuosa* and rye absorb iron slowly, hemp a great deal more rapidly.

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